

# **EXHIBIT A**

**A. '434 Patent, (Ex. B)**

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"DNA construct" (Asserted Claims 1-2, 5, 8)</p> <p><b>Identified by Defendants</b></p>	<p>To the extent the preamble is limiting: Plain and ordinary meaning, which is assembly of DNA molecules linked together</p> <p><b>Intrinsic Evidence:</b></p> <p>'434 patent cl. 1 ("A DNA construct comprising: a first, second, third, and fourth expression cassette . . . wherein the four cassettes are flanked by SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end.")</p> <p>'434 patent, 9:40-48 ("A DNA construct is an assembly of DNA molecules linked together that provide one or more expression cassettes. The DNA construct may be a plasmid . . . or a DNA construct may be a linear assembly of DNA molecules, such as an expression cassette.")</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	<p>A DNA construct comprising the first, second, third, and fourth expression cassettes (recited in claim 1) flanked by SEQ ID NO. 27 on the 5' end and SEQ ID NO. 28 on the 3' end.</p> <p><b>Intrinsic Evidence:</b></p> <p>February 4, 2013 Non-Final Rejection (Ex. H), at 6-9; May 2, 2013 Response to Office Action (Ex. I), at 15; July 1, 2013 Notice of Allowance (Ex. J), at 3-4.</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the DNA construct is defined by both the identity and specific locations of the expression cassettes, and the flanking sequences, the specific locations identified by the junction sequences unique to the event. The proposed construction is in accordance with the Examiner's amendment that defined the DNA construct as including the flanking sequences and not just the transgene insert.</p>



'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p data-bbox="199 235 619 267">"flanked by" (Asserted Claim 1)</p> <p data-bbox="199 308 541 341"><b>Identified by Defendants</b></p>	<p data-bbox="770 235 1325 300">Plain and ordinary meaning, which is joined or connected at the side to</p> <p data-bbox="770 349 1035 381"><b>Intrinsic Evidence:</b></p> <p data-bbox="770 414 1325 633">'434 patent, cl. 1 ("A DNA construct comprising: a first, second, third and fourth expression cassette . . . wherein the four cassettes are flanked by SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end.")</p> <p data-bbox="770 673 1325 958">'434 patent, 2:32-36 ("As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known.")</p> <p data-bbox="770 998 1325 1323">'434 patent, 8:25-30 ("A 'junction' is a point where two (2) specific DNA fragments join. For example, a junction exists where insert DNA joins flanking DNA. A junction point also exists in a transformed organism where two (2) DNA fragments join together in a manner that is modified from that found in the native organism.")</p>	<p data-bbox="1341 235 1507 267">Adjacent to.</p> <p data-bbox="1341 308 1608 341"><b>Intrinsic Evidence:</b></p> <p data-bbox="1341 381 1896 1364">'434 Patent at 2:35-36 ("flanking DNA adjacent to the inserted heterologous DNA"); <i>id.</i> at 8:11-19 ("A 'flanking region' or 'flanking sequence' as used herein refers to a sequence of at least 20 bp, preferably at least 50 bp, and up to 5000 bp, which is located either immediately upstream of and contiguous with or immediately downstream of and contiguous with the original foreign insert DNA molecule. Transformation procedures leading to random integration of the foreign DNA will result in transformants containing different flanking regions characteristic and unique for each transformant"); <i>id.</i> at 10:42-43 ("flanking sequence immediately adjacent to the inserted DNA"); <i>id.</i> at 16:35-36 ("flanking sequence adjacent to the insertion site of inserted heterologous DNA"); <i>id.</i> at 17:9-10 ("adjacent flanking DNA sequence"); <i>id.</i> at 17:12-13 ("using one primer in the inserted sequence and one in the adjacent flanking sequence"); <i>id.</i> at 17:23-24 ("an oligonucleotide is designed that overlaps the adjacent DNA and insert DNA junction").</p>

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	<p>'434 patent, 10:40-49 ("The term 'event' also refers to DNA from the original transformant comprising the inserted DNA and flanking sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.")</p> <p>'434 patent, 16:29-38 ("For example, to determine whether a corn plant resulting from a sexual cross contains transgenic event genomic DNA from the corn plant of the invention, DNA extracted from the corn plant tissue sample may be subjected to a nucleic acid amplification method using a DNA primer pair that includes a first primer derived from flanking sequence adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA.")</p> <p>'434 patent, 17:5-17 ("The amplicon produced by these methods may be</p>	<p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the claimed plant is a DP4114 event plant by requiring the two flanking sequences to be located at positions unique to DP4114 event which was the basis for allowance of the claims. The flanking sequences define the position of the transgene in the genome, which was the alleged patentable characteristic of the claim. To construe the claim differently would impermissibly broaden the scope of the claim to cover a number of possibilities that are not enabled by the specification.</p>

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	<p>detected by a plurality of techniques, including, but not limited to, Genetic Bit Analysis . . . where a DNA oligonucleotide is designed which overlaps both the adjacent flanking DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking sequence) a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	
<p>“the genotype of the corn event DP-004114-3” (Asserted Claims 5-6)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is the genetic constitution of the corn event DP-004114-3</p> <p>Corteva's proposed construction of “corn event DP-004114-3” is provided below.</p> <p><b>Intrinsic Evidence:</b></p>	<p>The complete sequence of the insert and flanking regions of event DP-004114-3, as claimed in claim 1 and as disclosed in SEQ ID NO: 6, which includes the four cassettes disclosed in Claim 1 flanked by SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end.</p>

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	<p>'434 patent, cl. 1 ("A DNA construct comprising: a first, second, third and fourth expression cassette, wherein said first expression cassette in operable linkage comprises: (a) a maize ubiquitin promoter; (b) a 5' untranslated exon of a maize ubiquitin gene; (c) a maize ubiquitin first intron; (d) a Cry1F encoding DNA molecule; and (e) a poly(A) addition signal from ORF 25 terminator; said second expression cassette in operable linkage comprises: (1) a maize ubiquitin promoter; (2) a 5' untranslated exon of a maize ubiquitin gene; (3) a maize ubiquitin first intron; (4) a Cry34Ab1 encoding DNA molecule; and (5) a PinII transcriptional terminator; said third expression cassette in operable linkage comprises; (i) a wheat peroxidase promoter; (ii) a Cry35Ab1 encoding DNA molecule; and (iii) a PinII transcriptional terminator; and said fourth expression cassette in operable linkage comprises; (a) a CaMV 35S promoter; (b) a pat encoding DNA molecule; and (c) a 3' transcriptional terminator from CaMV 35S; wherein the four cassettes are flanked by SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end.")</p> <p>'434 patent, cl. 5 ("A corn plant comprising the genotype of the corn event DP-004114-</p>	<p><b>Intrinsic Evidence:</b></p> <p>'434 Patent at 6:34-41 ("Compositions of this disclosure include seed deposited as Patent Deposit No. PTA-11506 and plants, plant cells, and seed derived therefrom"); <i>id.</i> at 83:33-36 (Claim 5: "A corn plant comprising the genotype of the corn event DP-004114-3 deposited with American Type Culture Collection (ATCC) under Accession No. PTA-11506, wherein said genotype comprises the DNA construct of claim 1."); <i>id.</i> at 83:37-40 (Claim 6: "A corn plant comprising the genotype of the corn event DP-004114-3, wherein a representative sample of seed of said corn event has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506"); February 4, 2013 Non-Final Rejection (Ex. H), at 6-9; May 2, 2013 Response to Office Action (Ex. I), at 15; July 1, 2013 Notice of Allowance (Ex. J), at 3-4.</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the genotype is of event DP4114 defined by both the identity and specific locations of the expression cassettes, and flanking sequences with the specific junction sequences unique to the event. The</p>

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	<p>3 deposited with American Type Culture Collection (ATCC) under Accession No. PTA-11506, wherein said genotype comprises the DNA construct of claim 1.”)</p> <p>'434 patent, cl. 6 (“A corn plant comprising the genotype of the corn event DP-004114-3, wherein a representative sample of seed of said corn event has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, cl. 8 (“A seed comprising corn event DP-004114-3, wherein said seed comprises the DNA construct of claim 1, wherein a representative sample of corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, cl. 13 (“An amplicon comprising the nucleic acid sequence selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28, and full length complements thereof.”)</p> <p>'434 patent, cl. 14 (“A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected</p>	<p>specific flanking sequences directly adjacent to (i.e., flanking) the expression cassettes comprise the unique junction sequences unique to the event.</p>

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	<p>from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, cl. 15 (“The biological sample of claim 14, wherein said biological sample comprise plant, tissue, or seed of transgenic corn event DP-004114-3.”)</p> <p>'434 patent, cl. 16 (“The biological sample of claim 15, wherein said biological sample is a DNA sample extracted from the transgenic corn plant event DP-004114-3, and wherein said DNA sample comprises one or more of the nucleotide sequences selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28, and the complement thereof.”)</p> <p>'434 patent, cl. 21 (“A method of producing hybrid corn seeds comprising: (a) planting seeds of a first inbred corn line comprising the DNA construct of claim 1 and seeds of a second inbred line having a</p>	

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	<p>genotype different from the first inbred corn line; (b) cultivating corn plants resulting from said planting until time of flowering; (c) emasculating said flowers of plants of one of the corn inbred lines; (d) sexually crossing the two different inbred lines with each other; and (e) harvesting the hybrid seed produced thereby.”)</p> <p>'434 patent, Abstract (“The invention provides DNA compositions that relate to transgenic insect resistant maize plants. Also provided are assays for detecting the presence of the maize DP-004114-3 event based on the DNA sequence of the recombinant construct inserted into the maize genome and the DNA sequences flanking the insertion site. Kits and conditions useful in conducting the assays are provided.”)</p> <p>'434 patent, 2:52-56 (“Maize event DP-004114-3 was produced by <i>Agrobacterium</i>-mediated transformation with plasmid PHP27118. This event contains the cry1F, cry34Ab1, cry35Ab1, and pat gene cassettes, which confer resistance to certain lepidopteran and coleopteran pests, as well as tolerance to phosphinothricin.”)</p>	

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	<p>'434 patent, 3:41-51 ("According to another embodiment of the invention, compositions and methods are provided for identifying a novel corn plant designated DP-004114-3. The methods are based on primers or probes which specifically recognize the 5' and/or 3' flanking sequence of DP-004114-3. DNA molecules are provided that comprise primer sequences that when utilized in a PCR reaction will produce amplicons unique to the transgenic event DP-004114-3. The corn plant and seed comprising these molecules is an embodiment of this invention. Further, kits utilizing these primer sequences for the identification of the DP-004114-3 event are provided.")</p> <p>'434 patent, 4:15-27 ("More specifically, a method for detecting the presence of a DNA molecule corresponding to the DP-004114-3 event in a sample, such methods consisting of (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that consists of sequences that are unique to the event, e.g., junction sequences, wherein said DNA probe molecule hybridizes under stringent hybridization conditions with DNA extracted from corn event DP-004114-3 and does not hybridize under the stringent hybridization conditions with a</p>	



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	<p>control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.”)</p> <p>'434 patent, 4:28-30 (“In addition, a kit and methods for identifying event DP-004114-3 in a biological sample which detects a DP-004114-3 specific region are provided.”)</p> <p>'434 patent, 4:31-36 (“DNA molecules are provided that comprise at least one junction sequence of DP-004114-3; wherein a junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the corn cell flanking the insertion site, i.e. flanking DNA, and is diagnostic for the DP-004114-3 event.”)</p> <p>'434 patent, 5:63-6:2 (“Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger et al., <i>Glossary of Genetics: Classical and Molecular</i>, 5<sup>th</sup> edition, Springer-Verlag, New York, 1991; and Lewin, <i>Genes V</i>, Oxford University Press: New York, 1994”)</p>	

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	<p>'434 patent, 7:12-17 ("As used herein, the term 'DP-004114-3 specific' refers to a nucleotide sequence which is suitable for discriminatively identifying event DP-004114-3 in plants, plant material, or in products such as, but not limited to, food or feed products (fresh or processed) comprising, or derived from plant material.")</p> <p>'434 patent, 8:31-36 ("Two junction sequences set forth in this disclosure are the junction point between the maize genomic DNA and the 5' end of the insert as set forth in SEQ ID NO: 27, and the junction point between the 3' end of the insert and maize genomic DNA as set forth in SEQ ID NO: 28.")</p> <p>'434 patent, 10:14-19 ("It is to be understood that as used herein the term 'transgenic' includes any cell, cell line, callus, tissue, plant part, or plant, the genotype of which has been altered by the presence of a heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic.")</p>	

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	<p>'434 patent, 10:26-32 ("A transgenic 'event' is produced by transformation of plant cells with a heterologous DNA construct(s), including a nucleic acid expression cassette that comprises a transgene of interest, the regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location.")</p> <p>'434 patent, 10:34-35 ("At the genetic level, an event is part of the genetic makeup of a plant")</p> <p>'434 patent, 10:35-37 ("The term 'event' also refers to progeny produced by a sexual outcross between the transformant and another variety that include the heterologous DNA.")</p> <p>'434 patent, 10:40-49 ("The term 'event' also refers to DNA from the original transformant comprising the inserted DNA and flanking sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the</p>	

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	<p>original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.”)</p> <p>'434 patent, 17:34-38 (“Fluorescence polarization as described by Chen . . . is also a method that can be used to detect an amplicon of the invention. Using this method an oligonucleotide is designed which overlaps the flanking and inserted DNA junction.”)</p> <p>'434 patent, 17:51-53, (“Briefly, a FRET oligonucleotide probe is designed which overlaps the flanking and insert DNA junction.”); 17:64-66 (“Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking and insert DNA junction.”)</p> <p>'434 patent, Ex. 4, <i>e.g.</i>, 32:33 (“Genotype Confirmation Via Event-Specific PCR Analysis”), 32:40-45 (“Real-time PCR was performed on each DNA sample . . and primer sets . . . were designed to detect a target sequence from 4114 maize.”)</p> <p>'434 patent, 34:4-17 (“Sequence information obtained from inverse PCR was subjected to BLASTn analysis and showed a match to the maize BAC clone AC211214 from the NCBI . . . GenBank</p>	

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	<p>nucleotide database. This sequence was then used to design primers that spanned the 5' and 3' insert/genomic junctions in 4114 maize. The PCR products generated from four 4114 maize plants were cloned and sequenced to verify the 5' and 3' insert/genomic junctions and the genomic border regions. In addition, to demonstrate that the identified 5' and 3' genomic border regions were of maize origin, PCR was performed on 4114 maize and control maize plants within the genomic regions. Each PCR fragment was directly sequenced to verify its identity of maize origin.”)</p> <p>'434 patent, Figs. 2, 5</p> <p>'434 patent, SEQ ID NO: 6 (“The complete sequence of the insert and flanking regions of event DP-004114-3”)</p> <p>'434 patent, SEQ ID NO: 27 (“5' Junction Sequence of event DP-004114-3”)</p> <p>'434 patent, SEQ ID NO: 28 (“3' Junction Sequence of event DP-004114-3”)</p> <p>U.S. Patent Application No. 12/970,052 Prosecution History, Feb. 4, 2013 Office Action (Ex. H) at 11 (“The following is a statement of reasons for the indication of</p>	

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	<p>allowable subject matter: The corn event DP-004114-3 is not known in the prior art. The event is described as a DNA construct comprising four operably linked cassettes comprising three Cry toxins and a phosphinothricin resistance gene, wherein the construct is flanked SEQ ID NO: 27 and 28 (20mers) in a corn plant.”)</p> <p>Rieger et al., <i>Glossary of Genetics: Classical and Molecular</i>, 5th ed., Springer Verlag, New York, 1991 (Ex. K) at 230 (“[t]he genetic constitution in respect to the alleles at one or a few pairs of genetic loci under observation.”), <i>id.</i> (“the sum total of the genetic information (genes) contained in the linkage structures (chromosomes) of the pro- and eukaryotes, as distinguished from their phenotype.”);</p> <p>Lewin, <i>Genes</i> V, Oxford University Press: New York, 1994 (Ex. L) at 1243 (“Genotype is the genetic constitution of an organism.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	

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<p data-bbox="199 235 753 267">"plant" (Asserted Claims 2-7, 9, 14-15)</p> <p data-bbox="199 308 541 341"><b>Identified by Defendants</b></p>	<p data-bbox="770 235 1327 332">Plain and ordinary meaning, which is organism belonging to the kingdom Plantae</p> <p data-bbox="770 381 1037 414"><b>Intrinsic Evidence:</b></p> <p data-bbox="770 454 1327 1218"><i>E.g.</i>, '434 patent, cl. 5 ("A corn plant comprising the genotype of the corn event DP-004114-3 deposited with American Type Culture Collection (ATCC) under Accession No. PTA-11506, wherein said genotype comprises the DNA construct of claim 1."), cl. 14 ("A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.")</p> <p data-bbox="770 1258 1327 1396">'434 patent, cl. 21 ("A method of producing hybrid corn seeds comprising: (a) planting seeds of a first inbred corn line comprising the DNA construct of claim 1</p>	<p data-bbox="1344 235 1728 267">An event DP-004114-3 plant.</p> <p data-bbox="1344 308 1608 341"><b>Intrinsic Evidence:</b></p> <p data-bbox="1344 381 1900 706">'434 Patent at 83:27-28 (Claim 1: "wherein the four cassettes are flanked by SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end"); <i>id.</i> at 83:29 ("A plant comprising the DNA construct of claim 1"). February 4, 2013 Non-Final Rejection (Ex. H), at 6-9; May 2, 2013 Response to Office Action (Ex. I), at 15; July 1, 2013 Notice of Allowance (Ex. J), at 3-4.</p> <p data-bbox="1344 747 1850 779"><b>Why Resolution Makes a Difference:</b></p> <p data-bbox="1344 820 1900 1031">The proposed construction is consistent with the limitations in the claims that uniquely define an event DP-004114-3 plant and ensures that the scope of the claims is consistent with the breadth to which it was narrowed during prosecution.</p>

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	<p>and seeds of a second inbred line having a genotype different from the first inbred corn line; (b) cultivating corn plants resulting from said planting until time of flowering; (c) emasculating said flowers of plants of one of the corn inbred lines; (d) sexually crossing the two different inbred lines with each other; and (e) harvesting the hybrid seed produced thereby.”)</p> <p><i>E.g.</i>, '434 patent, cl. 25 (“The method of claim 21 further comprising backcrossing the second generation progeny plant of step (d) that comprises corn event DP-004114-3 DNA, deposited under Accession No. PTA-11506 with American Type Culture Collection (ATCC), to the parent plant that lacks the corn event DP-004114-3 DNA, thereby producing a backcross progeny plant that is resistant to at least western corn rootworm.”)</p> <p><i>E.g.</i>, '434 patent, cl. 26 (“A method for producing a corn plant resistant to at least corn rootworm, said method comprising: (a) sexually crossing a first parent corn plant with a second parent corn plant, wherein said first or second parent corn plant is a corn event DP-004114-3, deposited under Accession No. PTA-11506 with American Type Culture Collection (ATCC), plant, thereby</p>	



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	<p>producing a plurality of first generation progeny plants; (b) selecting a first generation progeny plant that is resistant to at least corn rootworm infestation; (c) backcrossing the first generation progeny plant of step (b) with the parent plant that lacks corn event DP-004114-3 DNA, thereby producing a plurality of backcross progeny plants; and (d) selecting from the backcross progeny plants, a plant that is resistant to at least corn rootworm infestation; wherein the selected backcross progeny plant of step (d) comprises SEQ ID NO: 6.”)</p> <p><i>E.g.</i>, '434 patent, 1:15-18 (“Embodiments of the present invention relate to the field of plant molecular biology, specifically embodiment of the invention relate to DNA constructs for conferring insect resistance to a plant.”), 1:52-56 (“The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site.”)</p> <p>'434 patent, 10:66-11:1 (“As used herein, the term ‘plant’ includes reference to whole plants, plant organs (e.g., leaves,</p>	

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	<p>stems, roots, etc.), seeds, plant cells, and progeny of same.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	
<p>“corn event DP-004114-3” (Asserted Claims 5-6, 8, 14-15)</p> <p><b>Identified by Defendants</b></p>	<p>“a Cry1F-encoding expression cassette, a Cry34Ab1-encoding expression cassette, a Cry35Ab1-encoding expression cassette, and a pat-encoding expression cassette, located between SEQ ID NO: 27 at the 5’ end and SEQ ID NO: 28 at the 3’ end”</p> <p><b>Intrinsic Evidence:</b></p> <p>’434 patent, cl. 1 (“A DNA construct comprising: a first, second, third and fourth expression cassette, wherein said first expression cassette in operable linkage comprises: (a) a maize ubiquitin promoter; (b) a 5’ untranslated exon of a maize ubiquitin gene; (c) a maize ubiquitin first intron; (d) a Cry1F encoding DNA molecule; and (e) a poly(A) addition signal from ORF 25 terminator; said second expression cassette in operable linkage comprises: (1) a maize ubiquitin promoter; (2) a 5’ untranslated exon of a maize ubiquitin gene; (3) a maize ubiquitin first</p>	<p>The complete sequence of the insert and flanking regions of event DP-004114-3, as disclosed in SEQ ID NO: 6, which includes the four cassettes disclosed in Claim 1 flanked by SEQ ID NO: 27 at the 5’ end and SEQ ID NO: 28 at the 3’ end.</p> <p><b>Intrinsic Evidence:</b></p> <p>’434 Patent at 83:37-40 (Claim 6: “A corn plant comprising the genotype of the corn event DP-004114-3, wherein a representative sample of seed of said corn event has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506”).</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the event is defined by both the identity and specific locations of the expression cassettes, wherein the specific locations are</p>

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	<p>intron; (4) a Cry34Ab1 encoding DNA molecule; and (5) a PinII transcriptional terminator; said third expression cassette in operable linkage comprises; (i) a wheat peroxidase promoter; (ii) a Cry35Ab1 encoding DNA molecule; and (iii) a PinII transcriptional terminator; and said fourth expression cassette in operable linkage comprises; (a) a CaMV 35S promoter; (b) a pat encoding DNA molecule; and (c) a 3' transcriptional terminator from CaMV 35S; wherein the four cassettes are flanked by SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end.")</p> <p>'434 patent, cl. 5 ("A corn plant comprising the genotype of the corn event DP-004114-3 deposited with American Type Culture Collection (ATCC) under Accession No. PTA-11506, wherein said genotype comprises the DNA construct of claim 1.")</p> <p>'434 patent, cl. 6 ("A corn plant comprising the genotype of the corn event DP-004114-3, wherein a representative sample of seed of said corn event has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.")</p> <p>'434 patent, cl. 8 ("A seed comprising corn event DP-004114-3, wherein said seed</p>	<p>identified by the junction sequences unique to the event that are in SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end.</p>

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	<p>comprises the DNA construct of claim 1, wherein a representative sample of corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, cl. 13 (“An amplicon comprising the nucleic acid sequence selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28, and full length complements thereof.”)</p> <p>'434 patent, cl. 14 (“A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, cl. 15 (“The biological sample of claim 14, wherein said biological sample</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>comprise plant, tissue, or seed of transgenic corn event DP-004114-3.”)</p> <p>'434 patent, cl. 16 (“The biological sample of claim 15, wherein said biological sample is a DNA sample extracted from the transgenic corn plant event DP-004114-3, and wherein said DNA sample comprises one or more of the nucleotide sequences selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28, and the complement thereof.”)</p> <p>'434 patent, Abstract (“The invention provides DNA compositions that relate to transgenic insect resistant maize plants. Also provided are assays for detecting the presence of the maize DP-004114-3 event based on the DNA sequence of the recombinant construct inserted into the maize genome and the DNA sequences flanking the insertion site. Kits and conditions useful in conducting the assays are provided.”)</p> <p>'434 patent, 2:52-56 (“Maize event DP-004114-3 was produced by <i>Agrobacterium</i>-mediated transformation with plasmid PHP27118. This event contains the cry1F, cry34Ab1, cry35Ab1, and pat gene cassettes, which confer resistance to certain lepidopteran and</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>coleopteran pests, as well as tolerance to phosphinothricin.”)</p> <p>'434 patent, 3:41-51 (“According to another embodiment of the invention, compositions and methods are provided for identifying a novel corn plant designated DP-004114-3. The methods are based on primers or probes which specifically recognize the 5’ and/or 3’ flanking sequence of DP-004114-3. DNA molecules are provided that comprise primer sequences that when utilized in a PCR reaction will produce amplicons unique to the transgenic event DP-004114-3. The corn plant and seed comprising these molecules is an embodiment of this invention. Further, kits utilizing these primer sequences for the identification of the DP-004114-3 event are provided.”)</p> <p>'434 patent, 4:15-27 (“More specifically, a method for detecting the presence of a DNA molecule corresponding to the DP-004114-3 event in a sample, such methods consisting of (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that consists of sequences that are unique to the event, e.g., junction sequences, wherein said DNA probe molecule hybridizes under stringent hybridization conditions with</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>DNA extracted from corn event DP-004114-3 and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.”)</p> <p>'434 patent, 4:28-30 (“In addition, a kit and methods for identifying event DP-004114-3 in a biological sample which detects a DP-004114-3 specific region are provided.”)</p> <p>'434 patent, 4:31-36 (“DNA molecules are provided that comprise at least one junction sequence of DP-004114-3; wherein a junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the corn cell flanking the insertion site, i.e. flanking DNA, and is diagnostic for the DP-004114-3 event.”)</p> <p>'434 patent, 7:12-17 (“As used herein, the term ‘DP-004114-3 specific’ refers to a nucleotide sequence which is suitable for discriminatively identifying event DP-004114-3 in plants, plant material, or in products such as, but not limited to, food or feed products (fresh or processed)</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>comprising, or derived from plant material.”)</p> <p>'434 patent, 8:31-36 (“Two junction sequences set forth in this disclosure are the junction point between the maize genomic DNA and the 5’ end of the insert as set forth in SEQ ID NO: 27, and the junction point between the 3’ end of the insert and maize genomic DNA as set forth in SEQ ID NO: 28.”)</p> <p>'434 patent, 10:34-35 (“At the genetic level, an event is part of the genetic makeup of a plant”)</p> <p>'434 patent, 10:35-37 (“The term ‘event’ also refers to progeny produced by a sexual outcross between the transformant and another variety that include the heterologous DNA.”)</p> <p>'434 patent, 10:40-49 (“The term ‘event’ also refers to DNA from the original tranformant comprising the inserted DNA and flanking sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the</p>	



'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.”)</p> <p>'434 patent, 17:34-38 (“Fluorescence polarization as described by Chen . . . is also a method that can be used to detect an amplicon of the invention. Using this method an oligonucleotide is designed which overlaps the flanking and inserted DNA junction.”)</p> <p>'434 patent, 17:51-53 (“Briefly, a FRET oligonucleotide probe is designed which overlaps the flanking and insert DNA junction.”); 17:64-66 (“Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking and insert DNA junction.”)</p> <p>'434 patent, 34:4-17 (“Sequence information obtained from inverse PCR was subjected to BLASTn analysis and showed a match to the maize BAC clone AC211214 from the NCBI . . . GenBank nucleotide database. This sequence was then used to design primers that spanned the 5' and 3' insert/genomic junctions in 4114 maize. The PCR products generated from four 4114 maize plants were cloned and sequenced to verify the 5' and 3' insert/genomic junctions and the genomic</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>border regions. In addition, to demonstrate that the identified 5' and 3' genomic border regions were of maize origin, PCR was performed on 4114 maize and control maize plants within the genomic regions. Each PCR fragment was directly sequenced to verify its identity of maize origin.”)</p> <p>'434 patent, Figs. 2, 5</p> <p>'434 patent, SEQ ID NO: 6 (“The complete sequence of the insert and flanking regions of event DP-004114-3”)</p> <p>'434 patent, SEQ ID NO: 27 (“5' Junction Sequence of event DP-004114-3”)</p> <p>'434 patent, SEQ ID NO: 28 (“3' Junction Sequence of event DP-004114-3”)</p> <p>U.S. Patent Application No. 12/970,052 Prosecution History, Feb. 4, 2013 Office Action (Ex. H) at 11 (“The following is a statement of reasons for the indication of allowable subject matter: The corn event DP-004114-3 is not known in the prior art. The event is described as a DNA construct comprising four operably linked cassettes comprising three Cry toxins and a phosphinothricin resistance gene, wherein</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>the construct is flanked SEQ ID NO: 27 and 28 (20mers) in a corn plant.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	
<p>“seed” (Asserted Claims 6, 8-9, 14-15)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is ripened ovule of a flowering plant that may develop into a new plant</p> <p><b>Intrinsic Evidence:</b></p> <p>'434 patent cl. 6 (“A corn plant comprising the genotype of the corn event DP-004114-3, wherein a representative sample of seed of said corn event has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, cl. 8 (“A seed comprising corn event DP-004114-3, wherein said seed comprises the DNA construct of claim 1, wherein a representative sample of corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p>	<p>Plain and ordinary meaning, which is a seed coat, food store, and plant embryo.</p> <p><b>Intrinsic Evidence:</b></p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the term retains its plain and ordinary meaning to a person of ordinary skill in the art. The plain and ordinary meaning helps clarify that the seed already contains a plant in embryo form rather than something that may become a plant. Construing this term is relevant to the infringement claim under 35 U.S.C. § 271(f)(2).</p>

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'434 patent, cl. 9 ("A corn plant, or part thereof, grown from the seed of claim 8.")</p> <p>'434 patent, cl. 10 ("A transgenic seed produced from the corn plant of claim 9, wherein the seed comprises corn event DP-004114-3.")</p> <p>'434 patent, cl. 11 ("A transgenic corn plant, or part thereof, grown from the seed of claim 10.")</p> <p>'434 patent, cl. 14 ("A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.")</p> <p>'434 patent, cl. 15 ("The biological sample of claim 14, wherein said biological sample comprise plant, tissue, or seed of transgenic corn event DP-004114-3.")</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'434 patent, 11:1-3 ("Parts of transgenic plants understood to be within the scope of the invention comprise, for example, plant cells, protoplasts, tissues, callus, embryos . . .")</p> <p>'434 patent, 11:9-10 ("As used herein, the term 'plant cell' includes, without limitation, seeds, suspension cultures, embryos . . .")</p> <p>'434 patent, Example 1, <i>e.g.</i>, 25:64-26:58 ("After three to five days on this medium, embryos were then transferred to selective medium that was stimulatory to maize somatic embryogenesis and contained bialaphos for selection of cells expressing the pat transgene. The medium also contained carbenicillin to kill any remaining <i>Agrobacterium</i>. After six to eight weeks on the selective medium, healthy, growing calli that demonstrated resistance to bialaphos were identified. The putative transgenic calli were subsequently regenerated to produce T0 plantlets.")</p> <p>'434 patent, Example 2, <i>e.g.</i>, 27:29-32 ("Test and control leaf samples (V5-V7) leaf stage) were harvested from plants grown at the DuPont Experimental Station</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>(Wilmington, Del.) from seed obtained from Pioneer Hi-Bred (Johnston, Iowa).")</p> <p>'434 patent, Example 4, <i>e.g.</i>, 32:21-27 ("The 4114 maize seed and the control seed were planted in growth chambers at the DuPont Experimental Station (Wilmington, Del.) to produce plant tissues used for this study. One seed was planted per pot, and the pot was uniquely identified. All plants were grown with light, temperature, and water regulated for healthy plant growth.")</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	
<p>"derived from" (Asserted Claim 14)</p> <p><b>Identified by Defendants</b></p>	<p>To the extent the preamble is limiting: Plain and ordinary meaning, which is formed or developed out of</p> <p><b>Intrinsic Evidence:</b></p> <p>'434 patent, cl. 14 ("A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement</p>	<p>Extracted or processed from.</p> <p><b>Intrinsic Evidence:</b></p> <p>'434 patent at 13:4-14 ("A 'kit' as used herein refers to a set of reagents for the purpose of performing the method embodiments of the invention, more particularly, the identification of event DP-004114-3 in biological samples. The kit of the invention can be used, and its components can be specifically adjusted,</p>

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, cl. 15 (“The biological sample of claim 14, wherein said biological sample comprise plant, tissue, or seed of transgenic corn event DP-004114-3.”)</p> <p>'434 patent, cl. 18 (“An extract derived from corn event DP-004114-3 plant, tissue, or seed and comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28 or the complement thereof, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, 5:17-21 (“A further embodiment of this invention relates to the DP-004114-3 corn plant or its parts, including, but not limited to, pollen, ovules, vegetative cells, the nuclei of</p>	<p>for purposes of quality control (e.g., purity of seed lots), detection of event DP-004114-3 in plant material, or material comprising or derived from plant material, such as but not limited to food or feed products. ‘Plant material’ as used herein refers to material which is obtained or derived from a plant”); <i>id.</i> at 83:62-84:4 (Claim 14: “A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction ensures that the scope of the claim is consistent with the subject matter of the claim, which is directed to obtaining biological material from a plant to detect the presence of event</p>

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>pollen cells, and the nuclei of egg cells of the corn plant DP-004114-3 and the progeny derived thereof.”)</p> <p>'434 patent, 6:34-36 (“Compositions of this disclosure include seed deposited as Patent Deposit No. PTA-11506 and plants, plant cells, and seed derived therefrom.”)</p> <p>'434 patent, 10:50-61 (“An insect resistant DP-004114-3 corn plant can be bred by first sexually crossing a first parental corn plant consisting of a corn plant grown from the transgenic DP-004114-3 corn plant and progeny thereof derived from transformation with the expression cassettes of the embodiments of the present invention that confers insect resistance, and a second parental corn plant that lacks insect resistance, thereby producing a plurality of first progeny plants; and then selecting a first progeny plant that is resistant to insect; and selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants an insect resistant plant.”)</p> <p><b>Why Resolution Makes a Difference:</b></p>	<p>DP-004114-3 using nucleic acid amplification or hybridization.</p>



'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>“wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof” (Asserted Claim 14)</p> <p><b>Identified by Plaintiffs</b></p>	<p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p> <p>This language should be interpreted as a Markush group, which is “wherein said sample comprises a nucleotide sequence comprising any of: SEQ ID NO: 27, the complement of SEQ ID NO: 27, SEQ ID NO: 28, or the complement of SEQ ID NO: 28”</p> <p><b>Intrinsic Evidence:</b></p> <p>'434 patent, cl. 14 (“A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.”)</p> <p>'434 patent, cl. 1 (“A DNA construct comprising: a first, second, third and fourth</p>	<p>Plain and ordinary meaning, which is “wherein the sample comprises in its DNA a nucleotide sequence selected from the group consisting of SEQ ID NO: 27, or the complement thereof, located at the 3' end of the insert and SEQ ID NO: 28, or the complement thereof, located at the 5' end of the insert.”</p> <p><b>Intrinsic Evidence:</b></p> <p>'434 Patent at 8:31-36 (“Two junction sequences set forth in this disclosure are the junction point between the maize genomic DNA and the 5' end of the insert as set forth in SEQ ID NO: 27, and the junction point between the 3' end of the insert and maize genomic DNA as set forth in SEQ ID NO: 28.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Inari believes that this term does not require construction, but if it does, reflects the specific positions of the flanking sequences relative to the inserted transgenes.</p>

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>expression cassette, wherein said first expression cassette in operable linkage comprises: (a) a maize ubiquitin promoter; (b) a 5' untranslated exon of a maize ubiquitin gene; (c) a maize ubiquitin first intron; (d) a Cry1F encoding DNA molecule; and (e) a poly(A) addition signal from ORF 25 terminator; said second expression cassette in operable linkage comprises: (1) a maize ubiquitin promoter; (2) a 5' untranslated exon of a maize ubiquitin gene; (3) a maize ubiquitin first intron; (4) a Cry34Ab1 encoding DNA molecule; and (5) a PinII transcriptional terminator; said third expression cassette in operable linkage comprises; (i) a wheat peroxidase promoter; (ii) a Cry35Ab1 encoding DNA molecule; and (iii) a PinII transcriptional terminator; and said fourth expression cassette in operable linkage comprises; (a) a CaMV 35S promoter; (b) a pat encoding DNA molecule; and (c) a 3' transcriptional terminator from CaMV 35S; wherein the four cassettes are flanked by SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end.”)</p> <p>'434 patent, 2:14-24 (“It would be advantageous to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene of interest. In</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>addition, a method for detecting a particular event would be helpful for complying with regulations requiring the pre-market approval and labeling of foods derived from recombinant crop plants, for example, or for use in environmental monitoring, monitoring traits in crops in the field, or monitoring products derived from a crop harvest, as well as for use in ensuring compliance of parties subject to regulatory or contractual terms.”)</p> <p>'434 patent, 2:25-36 (“It is possible to detect the presence of a transgene by any nucleic acid detection method known in the art including, but not limited to, the polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc., because for many DNA constructs, the coding region is interchangeable. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known.”)</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'434 patent, 3:41-51 ("According to another embodiment of the invention, compositions and methods are provided for identifying a novel corn plant designated DP-004114-3. The methods are based on primers or probes which specifically recognize the 5' and/or 3' flanking sequence of DP-004114-3. DNA molecules are provided that comprise primer sequences that when utilized in a PCR reaction will produce amplicons unique to the transgenic event DP-004114-3. The corn plant and seed comprising these molecules is an embodiment of this invention. Further, kits utilizing these primer sequences for the identification of the DP-004114-3 event are provided.")</p> <p>'434 patent, 3:52-61 ("An additional embodiment of the invention relates to the specific flanking sequence of DP-004114-3 described herein, which can be used to develop specific identification methods for DP-004114-3 in biological samples. More particularly, the invention relates to the 5' and/or 3' flanking regions of DP-004114-3 which can be used for the development of specific primers and probes. A further embodiment of the invention relates to identification methods for the presence of DP-004114-3 in biological samples based</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>on the use of such specific primers or probes.”)</p> <p>'434 patent, 4:31-36 (“DNA molecules are provided that comprise at least one junction sequence of DP-004114-3; wherein a junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the corn cell flanking the insertion site, i.e. flanking DNA, and is diagnostic for the DP-004114-3 event.”)</p> <p>'434 patent, 4:56-63 (“Another embodiment of the invention further relates to a DNA detection kit for identifying maize event DP-004114-3 in biological samples. The kit comprises a first primer which specifically recognizes the 5' or 3' flanking region of DP-004114-3, and a second primer which specifically recognizes a sequence within the foreign DNA of DP-004114-3, or within the flanking DNA, for use in a PCR identification protocol.”)</p> <p>'434 patent, 4:63-5:3 (“A further embodiment of the invention relates to a kit for identifying event DP-004114-3 in biological samples, which kit comprises a specific probe having a sequence which corresponds or is complementary to, a</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>sequence having between 80% and 100% sequence identity with a specific region of event DP-004114-3. The sequence of the probe corresponds to a specific region comprising part of the 5' or 3' flanking region of event DP-004114-3.”)</p> <p>'434 patent, 8:31-36 (“Two junction sequences set forth in this disclosure are the junction point between the maize genomic DNA and the 5' end of the insert as set forth in SEQ ID NO: 27, and the junction point between the 3' end of the insert and maize genomic DNA as set forth in SEQ ID NO: 28.”)</p> <p>'434 patent, 12:44-52 (“In an embodiment of the invention the specific probe is a sequence which, under optimized conditions, hybridizes specifically to a region within the 5' or 3' flanking region of the event and also comprises a part of the foreign DNA contiguous therewith.”)</p> <p>'434 patent, Figs. 2, 5</p> <p>'434 patent, SEQ ID NO: 6 (“The complete sequence of the insert and flanking regions of event DP-004114-3”)</p> <p>'434 patent, SEQ ID NO: 27 (“5' Junction Sequence of event DP-004114-3”)</p>	

'434 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'434 patent, SEQ ID NO: 28 ("3' Junction Sequence of event DP-004114-3")</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The legal significance of Markush-group language in a claim ("selected from the group consisting of") is likely to be confusing to a jury absent explanation by the Court in its construction.</p>	

**B. '246 Patent, (Ex. C)**

'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"seed" (Asserted Claims 6, 8, 12-13)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is ripened ovule of a flowering plant that may develop into a new plant</p> <p><b>Intrinsic Evidence:</b></p> <p>'246 patent, 11:19-30 ("Parts of transgenic plants understood to be within the scope of the invention comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, leaves, and roots originating in transgenic plants or their progeny previously transformed with a DNA molecule of the invention and therefore consisting at least in part of transgenic cells, are also an embodiment of the present invention. As used herein, the term "plant cell" includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores.")</p> <p>'246 patent, Example 1, <i>e.g.</i>, 20:16-21 ("Specifically, the immature embryos were cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus was then regenerated into plants (step 5: the regeneration step), and, specifically, calli</p>	<p>Plain and ordinary meaning, which is a seed coat, food store, and plant embryo.</p> <p><b>Intrinsic Evidence:</b></p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the term retains its plain and ordinary meaning to a person of ordinary skill in the art. The plain and ordinary meaning helps clarify that the seed already contains a plant in embryo form rather than something that may become a plant. Construing this term is relevant to the infringement claim under 35 U.S.C. § 271(f)(2).</p>



'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>grown on selective medium were cultured on solid medium to regenerate the plants.”)</p> <p>'246 patent, Example 2, <i>e.g.</i>, 21:7-16 (“Corn seed for event DAS-59122-7 and unmodified control seed (Hi-II and PH09B) were planted in growth chambers at the DuPont Experimental Station (Wilmington, Del.) to produce sufficient numbers of plants for DNA analysis. For characterization of event DAS-59122-7, ten (10) T1S2 seeds were planted. Ten (10) seeds were also planted for each unmodified control line. One (1) seed was planted per pot, and the pot was uniquely identified. Planting and growing conditions were conducive to healthy plant growth including regulated light and water.”)</p> <p>U.S. Patent Publication No. 2006/0141495 A1 (cited on face of '246 patent) (Ex. M), [0019] (“A further aspect of this invention provides corn plants, including plant parts such as oil, progeny seeds, protein, etc. from corn plant produced by such marker assisted breeding methods.”)</p> <p><b>Why Resolution Makes a Difference:</b></p>	

'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.	
<p data-bbox="205 381 611 414">"linked" (Asserted Claims 1, 3)</p> <p data-bbox="205 456 541 488"><b>Identified by Defendants</b></p>	<p data-bbox="774 381 1325 451">Plain and ordinary meaning, which is joined or connected</p> <p data-bbox="774 493 1037 526"><b>Intrinsic Evidence:</b></p> <p data-bbox="774 568 1325 1399">'246 patent, cl. 1 ("A corn plant comprising in its genome a DNA construct linked to at least one flanking region, wherein: (a) said flanking region comprises a nucleotide sequence selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO: 19 and the nucleotide sequence set forth in SEQ ID NO: 20; (b) said DNA construct comprises a first, a second, and a third expression cassette; (c) said first expression cassette comprises in operable linkage (i) a maize ubiquitin promoter, (ii) a 5' untranslated exon of a maize ubiquitin gene, (iii) a maize ubiquitin first intron, (iv) a Cry34Ab1 encoding DNA molecule, and (v) a PinII transcriptional terminator; (d) said second expression cassette comprises in operable linkage (vi) a wheat peroxidase promoter, (vii) a Cry35Ab1 encoding DNA molecule, and (viii) a PinII transcriptional terminator; and (e) said third expression cassette comprises in</p>	<p data-bbox="1348 381 1570 414">Contiguous with.</p> <p data-bbox="1348 456 1610 488"><b>Intrinsic Evidence:</b></p> <p data-bbox="1348 531 1898 743">'246 Patent at 10:9-12 ("Operably linked' means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame.")</p> <p data-bbox="1348 786 1850 818"><b>Why Resolution Makes a Difference:</b></p> <p data-bbox="1348 860 1898 1073">This construction clarifies that the disclosed sequences are adjacent as in the patent disclosure of flanking sequences and not arguably joined or connected in other manner, e.g., by virtue of being in the same chromosome.</p>

'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>operable linkage (ix) a cauliflower mosaic virus (CaMV) 35S promoter; (x) a pat encoding DNA molecule; and (xi) a 3' transcriptional terminator from CaMV 35S.")</p> <p>'246 patent, 10:8-19 ("“Operably linked’ means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. Operably linked is intended to indicate a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes or multiple DNA constructs.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	

'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p data-bbox="199 235 753 305">"plant" (Asserted Claims 1, 3-4, 6, 8, 10-11)</p> <p data-bbox="199 345 541 378"><b>Identified by Defendants</b></p>	<p data-bbox="770 235 1327 337">Plain and ordinary meaning, which is organism belonging to the kingdom Plantae</p> <p data-bbox="770 378 1037 410"><b>Intrinsic Evidence:</b></p> <p data-bbox="770 451 1327 1399">'246 patent, cl. 1 ("A corn plant comprising in its genome a DNA construct linked to at least one flanking region, wherein: (a) said flanking region comprises a nucleotide sequence selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO: 19 and the nucleotide sequence set forth in SEQ ID NO: 20; (b) said DNA construct comprises a first, a second, and a third expression cassette; (c) said first expression cassette comprises in operable linkage (i) a maize ubiquitin promoter, (ii) a 5' untranslated exon of a maize ubiquitin gene, (iii) a maize ubiquitin first intron, (iv) a Cry34Ab1 encoding DNA molecule, and (v) a PinII transcriptional terminator; (d) said second expression cassette comprises in operable linkage (vi) a wheat peroxidase promoter, (vii) a Cry35Ab1 encoding DNA molecule, and (viii) a PinII transcriptional terminator; and (e) said third expression cassette comprises in operable linkage (ix) a cauliflower mosaic virus (CaMV) 35S promoter; (x) a pat encoding DNA molecule; and (xi) a 3'</p>	<p data-bbox="1344 235 1759 267">An event DAS59122 corn plant.</p> <p data-bbox="1344 308 1608 341"><b>Intrinsic Evidence:</b></p> <p data-bbox="1344 381 1900 889">'246 Patent at 77:27-32 ("A corn plant comprising in its genome a DNA construct linked to at least one flanking region, wherein: (a) said flanking region comprises a nucleotide sequence selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO: 19 and the nucleotide sequence set forth in SEQ ID NO: 20"); July 9, 2010 Final Rejection (Ex. N) at 2-3 ("The only such corn plants [as claimed in relevant claims, which contain SEQ ID NO: 19 and NO: 20,] taught in the specification are event DAS-59122-7 plants").</p> <p data-bbox="1344 930 1850 963"><b>Why Resolution Makes a Difference:</b></p> <p data-bbox="1344 1003 1900 1182">The proposed construction ensures that the scope of the claims is consistent with the limitations in the claims unique to event DAS59122 and the breadth to which it was narrowed during prosecution.</p>

'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>transcriptional terminator from CaMV 35S.”)</p> <p>'246 patent, cl. 2 (“The plant of claim 1, wherein said DNA construct comprises the nucleotide sequence set forth in SEQ ID NO: 24.”)</p> <p>'246 patent, cl. 3 (“The plant of claim 1, wherein said DNA construct is linked to a first and a second flanking region.”)</p> <p>'246 patent, cl. 4 (“The plant of claim 3, wherein said first flanking region comprises the nucleotide sequence set forth in SEQ ID NO: 19 and said second flanking region comprises the nucleotide sequence set forth in SEQ ID NO: 20.”)</p> <p>'246 patent, cl. 5 (“The plant of claim 4, wherein said DNA construct comprises the nucleotide sequence set forth in SEQ ID NO: 24.”)</p> <p>'246 patent, cl. 6 (“The plant of claim 1, wherein said plant is a seed.”)</p> <p>'246 patent, cl. 7 (“The plant of claim 2, wherein said plant is a seed.”)</p> <p>'246 patent, cl. 8 (“The plant of claim 4, wherein said plant is a seed.”)</p>	

'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'246 patent, cl. 9 ("The plant of claim 5, wherein said plant is a seed.")</p> <p>'246 patent, cl. 10 ("A plant comprising in its genome the nucleotide sequence set forth in SEQ ID NO: 23.")</p> <p>'246 patent, cl. 11 ("The plant of claim 10, wherein said plant is a corn plant.")</p> <p>'246 patent, cl. 14 ("The plant of claim 3, wherein said plant is a seed.")</p> <p><i>E.g.</i>, '246 patent, 1:15-18 ("Embodiments of the present invention relate to the field of plant molecular biology, specifically an embodiment of the invention relates to a DNA construct for conferring insect resistance to a plant."), 1:51-55 ("The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site.")</p> <p>'246 patent, 11:17-19 ("As used herein, the term 'plant' includes reference to whole plants, plant organs (e.g., leaves, stems,</p>	

'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>roots, etc.), seeds, plant cells, and progeny of same.”)</p> <p>'246 patent, 28:29-40 (“Combined, a total of 7343 bp of the T-DNA insert in event DAS-59122-7 was cloned and sequenced (from bp 2594 to bp 9936, see FIG. 1) and compared to the sequence of the transforming plasmid, PHP17662. Two nucleotide differences at bp 6526 and bp 6562 were observed in the non-translated wheat peroxidase promoter region of the T-DNA (see FIG. 1). . . Both the 3' and 5' end regions of the T-DNA insert were found to be intact, except for deletion of the last 22 bp at the 5' end and 25 bp at the 3' end encompassing the Right and Left T-DNA Border regions, respectively.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	
<p>“wherein: (a) said flanking region comprises a nucleotide sequence selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO: 19 and the nucleotide sequence set forth in SEQ ID NO: 20” (Asserted Claim 1)</p>	<p>This language should be interpreted as a Markush group, which is “wherein (a) said flanking region comprises a nucleotide sequence comprising SEQ ID NO: 19 or SEQ ID NO: 20”</p> <p><b>Intrinsic Evidence:</b></p>	<p>Wherein the nucleotide sequence SEQ ID NO: 19 is linked to and contiguous with the 5' end of the DNA construct and the nucleotide sequence SEQ ID NO: 20 is linked to and contiguous with the 3' end of the DNA construct.</p>

'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p><b>Identified by Plaintiffs</b></p>	<p>'246 patent cl. 1 ("A corn plant comprising in its genome a DNA construct linked to at least one flanking region, wherein: (a) said flanking region comprises a nucleotide sequence selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO: 19 and the nucleotide sequence set forth in SEQ ID NO: 20; (b) said DNA construct comprises a first, a second, and a third expression cassette; (c) said first expression cassette comprises in operable linkage (i) a maize ubiquitin promoter, (ii) a 5' untranslated exon of a maize ubiquitin gene, (iii) a maize ubiquitin first intron, (iv) a Cry34Ab1 encoding DNA molecule, and (v) a PinII transcriptional terminator; (d) said second expression cassette comprises in operable linkage (vi) a wheat peroxidase promoter, (vii) a Cry35Ab1 encoding DNA molecule, and (viii) a PinII transcriptional terminator; and (e) said third expression cassette comprises in operable linkage (ix) a cauliflower mosaic virus (CaMV) 35S promoter; (x) a pat encoding DNA molecule; and (xi) a 3' transcriptional terminator from CaMV 35S.")</p> <p>'246 patent, cl. 3 ("The plant of claim 1, wherein said DNA construct is linked to a first and a second flanking region.")</p>	<p><b>Intrinsic Evidence:</b></p> <p>'246 Patent at 4:1-5 ("More particularly, the invention relates to the 5' and/or 3' flanking regions of DAS 59122-7, SEQ ID NO: 19, 5' flanking and SEQ ID NO: 20, 3' flanking, respectively, which can be used for the development of specific primers and probes."); <i>id.</i> at 8:33-54 ("A 'flanking region' or 'flanking sequence' as used herein refers to a sequence ... which is located either immediately upstream of and contiguous with or immediately downstream of and contiguous with the original foreign insert DNA molecule. ... When recombinant DNA is introduced into a plant through traditional crossing, its flanking regions will generally not be changed. Transformants will also contain unique junctions between a piece of heterologous insert DNA and genomic DNA, or two (2) pieces of genomic DNA, or two (2) pieces of heterologous DNA. A 'junction' is a point where two (2) specific DNA fragments join. For example, a junction exists where insert DNA joins flanking DNA. A junction point also exists in a transformed organism where two (2) DNA fragments join together in a manner that is modified from that found in the native organism. 'Junction DNA' refers to DNA that comprises a junction point.")</p>



'246 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'246 patent, cl. 4 ("The plant of claim 3, wherein said first flanking region comprises the nucleotide sequence set forth in SEQ ID NO: 19 and said second flanking region comprises the nucleotide sequence set forth in SEQ ID NO: 20.")</p> <p>'246 patent, 3:65-4:5 ("An additional embodiment of the invention relates to the specific flanking sequences of DAS-59122-7 described herein, which can be used to develop specific identification methods for DAS-59122-7 in biological samples. More particularly, the invention relates to the 5' and/or 3' flanking regions of DAS-59122-7, SEQ ID NO: 19, 5' flanking and SEQ ID No: 20, 3' flanking, respectively, which can be used for the development of specific primers and probes.")</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The legal significance of Markush-group language in a claim ("selected from the group consisting of") is likely to be confusing to a jury absent explanation by the Court in its construction.</p>	<p><b>Why Resolution Makes a Difference:</b></p> <p>Inari believes that this term does not require construction, but if it does, Inari's construction reflects the specific positions of the flanking sequences relative to the inserted transgenes.</p>

**C. '522 Patent, (Ex. D)**

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity" (Asserted Claim 1)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning. A polynucleotide is a polymeric molecule composed of multiple nucleotides. A protein having aryloxyalkanoate dioxygenase activity is a protein with the ability to degrade or diminish the activity of an aryloxyalkanoate herbicide.</p> <p><b>Intrinsic Evidence:</b></p> <p>'522 patent, cl. 1 ("A plant cell comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 4.")</p> <p>'522 patent, cl. 2 ("A plant comprising a plurality of cells of claim 1.")</p> <p>'522 patent, cl. 3 ("A method of controlling weeds in a crop field, said method comprising applying an aryloxyalkanoate herbicide to said crop field, said crop field comprising a plurality of plants, each said plant comprising a plurality of plant cells of claim 1, wherein expression of said polynucleotide renders said plant resistant</p>	<p>Activity capable of degrading phenoxyacetate auxin and pyridyloxyacetate auxin herbicides to confer resistance to a plant to such herbicides.</p> <p><b>Intrinsic Evidence:</b></p> <p>'522 Patent at 3:56-58 ("The subject invention provides novel plants that are not only resistant to 2,4-D but also to pyridyloxyacetate herbicides."); <i>id.</i> at 4:29-35 ("the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and pyridyloxyacetate herbicides. No <math>\alpha</math>-ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of both the phenoxyacetate and pyridyloxyacetates auxin herbicides."); <i>id.</i> at 12:22-52 ("the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic auxin herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12) ... <i>Arabidopsis</i>, corn, tobacco, cotton, soybean, canola, and rice have been</p>

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>or tolerant to said aryloxyalkanoate herbicide.”)</p> <p>'522 patent, cl. 13 (“A seed comprising a plant cell of claim 1.”)</p> <p>'522 patent, 13:45-54 (“By ‘functional activity’ (or ‘active’) it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce ‘an effective amount’ of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide”)</p> <p>'522 patent, 22:37-40 (“In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner.”)</p> <p>'522 patent, Ex. 5 at, <i>e.g.</i>, 45:3 (“In Vitro Assays of AAD-12 Activity”)</p>	<p>transformed with AAD-12-containing constructs and have demonstrated high levels of resistance to both the phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to ‘plant optimized’ genes that encode proteins of the subject invention”) (emphasis in original); July 14, 2011 Non-Final Rejection (Ex. U), at 7-9; .October 14, 2011 Response to Office Action (Ex. V), at 6-8; March 27, 2012 Final Rejection (Ex. W), at 2.</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the claimed polynucleotide encodes a protein that confers to plants resistance to at least both phenoxyacetate auxin and pyridyloxyacetate auxin herbicides, which was the alleged surprising result that was the basis of overcoming a 35 U.S.C. § 103 rejection during prosecution. The construction of this term is relevant to Inari's invalidity claim.</p>

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'522 patent, Ex. 6 at, <i>e.g.</i>, 46:49-52 ("The activity of AAD-12 (v2) on 2,4-D was equivalent to that on (S)-dichlorprop indicating that the enzyme can process oxypropionate and oxyacetates effectively"); 46:64-66 ("These data show that AAD-12 (v2) is able to effectively degrade pyridyloxyalkanoate herbicides such as triclopyr.")</p> <p>Inari's Request for <i>Ex Parte</i> Reexamination of the '522 Patent Dated Nov. 18, 2022 (Ex. O) at 1 ("The Challenged Claims are generally directed to a plant cell with a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity. Plants are then composed of a plurality of these cells. Due to enhanced aryloxyalkanoate dioxygenase activity in the plant cells, plants composed of these cells are resistant or tolerant of aryloxyalkanoate-based herbicides.")</p> <p>Inari's Request for <i>Ex Parte</i> Reexamination of the '522 Patent Dated Nov. 18, 2022 (Ex. O) at 16 ("The main difference between claim 1 of the '522 patent and claim 1 of the '752 patent is that limitation [1.a] of the '522 patent claims a polynucleotide that encodes a protein with 'aryloxyalkanoate dioxygenase activity.' However, as is explained below and in the</p>	

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'752 patent, 'aryloxyalkanoate dioxygenase activity' is an 'activity that catalyzes degradation of a herbicidal compound selected from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin.'")</p> <p>Inari's Request for <i>Ex Parte</i> Reexamination of the '522 Patent Dated Nov. 18, 2022 (Ex. O) at 18 ("Therefore, a POSITA would have recognized, or at least found it obvious, that 'a polynucleotide that encodes a protein having aryloxyalkanoate activity' (as claimed in the '522 patent) is also 'a polynucleotide that encodes a protein having an activity that catalyzes degradation of a herbicidal compound selected from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin' (as claimed in the '752 patent)."</p> <p>U.S. Patent No. 10,167,483 (Ex. P), cl. 21 ("A polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein comprises SEQ ID NO:3.")</p>	

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>U.S. Patent No. 10,167,483 (Ex. P), cl. 22 (“A polynucleotide optimized for expression in a plant wherein said polynucleotide is operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein has SEQ ID NO:3.”)</p> <p>U.S. Patent No. 11,371,055 (Ex. Q), cl. 1 (“A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-12 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and a pyridyloxy auxin herbicide, further wherein said AAD-12 protein comprises: i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 2; and ii) an AAD-12 motif having the general formula of: <math>HX_{109}D(X)_{111-134}T(X)_{136-261}H(X)_{263-272}R</math>, wherein <math>X_{109}</math> represents a single amino acid at position 109, relative to the sequence of SEQ ID NO: 2; <math>(X)_{111-134}</math> represents a sequence of 24 amino acids; <math>(X)_{136-261}</math></p>	

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>represents a sequence of 126 amino acids; and (X)<sub>263-272</sub> represents a sequence of 10 amino acids.”)</p> <p>U.S. Patent No. 11,371,055 (Ex. Q), cl. 32 (“A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-12 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and a pyridyloxy auxin herbicide, further wherein said AAD-12 protein comprises: i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 2; and ii) an AAD-12 motif having the general formula of: HX<sub>109</sub>D(X)<sub>111-134</sub>T(X)<sub>136-261</sub>H(X)<sub>263-272</sub>R, wherein X<sub>109</sub> represents a single amino acid at position 109, relative to the sequence of SEQ ID NO: 2; (X)<sub>111-134</sub> represents a sequence of 24 amino acids; (X)<sub>136-261</sub> represents a sequence of 126 amino acids; and (X)<sub>263-272</sub> represents a sequence of 10 amino acids, wherein said AAD-12 motif has 90% sequence identity with corresponding amino acids of position 108 to 273 of SEQ ID NO: 2.”)</p> <p>U.S. Patent No. 11,371,055 (Ex. Q), cl. 33 (“A method of controlling at least one weed in a field, wherein said field has been</p>	

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>planted with seeds wherein cells of said seeds comprise a recombinant polynucleotide that encodes an AAD-12 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and a pyridyloxy auxin herbicide, further wherein said AAD-12 protein comprises: i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 2; and ii) an AAD-12 motif having the general formula of: <math>HX_{109}D(X)_{111-134}T(X)_{136-261}H(X)_{263-272}R</math>, wherein <math>X_{109}</math> represents a single amino acid at position 109, relative to the sequence of SEQ ID NO: 2; <math>(X)_{111-134}</math> represents a sequence of 24 amino acids; <math>(X)_{136-261}</math> represents a sequence of 126 amino acids; and <math>(X)_{263-272}</math> represents a sequence of 10 amino acids, wherein said method comprises applying to said field a pyridyloxy auxin herbicide.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Inari seeks to narrow this claim term, presumably to create a non-infringement and/or invalidity argument based on a requirement that the claimed activity affect two types of herbicides, but also that the claimed activity confer resistance to both herbicides to a plant. The construction is</p>	



'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	wrong because it is contrary to the intrinsic evidence. Moreover, it would not invalidate the claims, or negate infringement, even if it were adopted.	
<p>“seed” (Asserted Claim 13)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is ripened ovule of a flowering plant that may develop into a new plant</p> <p><b>Intrinsic Evidence:</b></p> <p>'522 patent, cl. 1 (“A plant cell comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO: 2 and SEQ ID NO: 4.”)</p> <p>'522 patent, cl. 2 (“A plant comprising a plurality of cells of claim 1.”)</p> <p>'522 patent, cl. 7 (“The method of claim 3 wherein said method further comprises applying said aryloxyalkanoate herbicide to said crop field prior to planting seeds in said field.”)</p> <p>'522 patent, cl. 8 (“The method of claim 3 wherein said method further comprises applying said aryloxyalkanoate herbicide</p>	<p>Plain and ordinary meaning, which is a seed coat, food store, and plant embryo.</p> <p><b>Intrinsic Evidence:</b></p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the term retains its plain and ordinary meaning to a person of ordinary skill in the art. The plain and ordinary meaning helps clarify that the seed already contains a plant in embryo form rather than something that may become a plant. Construing this term is relevant to the infringement claim under 35 U.S.C. § 271(f)(2).</p>

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>to said crop field after seeds are planted in said field but prior to emergence of said plants grown from said seeds.”)</p> <p>'522 patent, cl. 13 (“A seed comprising a plant cell of claim 1.”)</p> <p>'522 patent, 22:47-53 (“Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention.”)</p> <p>'522 patent, 25:66-26:1 (“The plants may then be grown to seed and said seed can be used to establish future generations.”)</p> <p>'522 patent, Example 7, <i>e.g.</i>, 48:30-36 (“Seeds were germinated and plants were grown . . . . Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.”)</p> <p><b>Why Resolution Makes a Difference:</b></p>	

'522 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.	

**D. '363 Patent, (Ex. E)**

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"seed" (Asserted Claims 7-8)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is ripened ovule of a flowering plant that may develop into a new plant</p> <p><b>Intrinsic Evidence:</b></p> <p>'363 patent, cl. 7 ("A soybean plant, wherein representative seed of said soybean plant has been deposited with the American Type Culture Collection under Accession No. PTA-12006.")</p> <p>'363 patent, cl. 8 ("A seed or a part of the plant of claim 7, wherein said seed or part comprises SEQ ID NO: 14.")</p> <p>'363 patent, 4:11-15 ("The invention also includes soybean plant cells and plant parts including, but are not limited to pollen, ovule, flowers, shoots, roots, and leaves, and nuclei of vegetative cells, pollen cells, seed and seed meal, and egg cells, that contain soybean event 9852.814.19.1")</p> <p>'363 patent, 7:32-38 ("The subject invention also includes a herbicide-tolerant soybean plant grown from a seed deposited with the ATCC Deposit No. identified in paragraph [0021]. The subject invention further includes parts of said plant, such as</p>	<p>Plain and ordinary meaning, which is a seed coat, food store, and plant embryo.</p> <p><b>Intrinsic Evidence:</b></p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the term retains its plain and ordinary meaning to a person of ordinary skill in the art. The plain and ordinary meaning helps clarify that the seed already contains a plant in embryo form rather than something that may become a plant. Construing this term is relevant to the infringement claim under 35 U.S.C. § 271(f)(2).</p>

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>leaves, tissue samples, seeds produced by said plant, pollen, and the like . . .")</p> <p>'363 patent, 7:59-62 ("An insect resistant/glufosinate-tolerant soybean plant of the subject invention can be bred by first sexually crossing a first parental soybean plant consisting of a soybean plant grown from seed of any one of the lines referred to herein . . .")</p> <p>U.S. Patent Application No. 13/559,177 Prosecution History, July 10, 2013 Non-Final Office Action (Ex. R) at 17 ("Claim 9 is directed to the seed of the soybean plant of claim 8. Because the claims do not required [sic] that the seed be grown from the deposited seeds and comprise the event, the term 'seeds' is given the broadest reasonable interpretation as encompassing the seeds obtained from the plant of any filial generation of the plants grown from the deposited seeds.")</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"plants" (Unasserted Claims 1 and 5)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is organisms belonging to the kingdom Plantae</p> <p><b>Intrinsic Evidence:</b></p> <p>'363 patent, cl. 1 ("A method of controlling insects, said method comprising exposing insects to insect resistant soybean plants, said soybean plants comprising SEQ ID NO: 14 . . .")</p> <p>'363 patent, cl. 5 ("A method of controlling weeds in a soybean crop, said method comprising applying glufosinate herbicide to the soybean crop, said soybean crop comprising soybean plants comprising SEQ ID NO: 14.")</p> <p>'363 patent, cl. 6 ("A method of breeding a soybean plant, said method comprising: crossing a first plant with a second soybean plant to produce a third soybean plant, said first plant comprising DNA comprising SEQ ID NO: 14; and assaying said third soybean plant for the presence of SEQ ID NO: 14.")</p> <p>'363 patent, cl. 7 ("A soybean plant, wherein representative seed of said soybean plant has been deposited with the</p>	<p>Event DAS81419 soybean plants.</p> <p><b>Intrinsic Evidence:</b></p> <p>'363 Patent at 63:65-66 (Claim 1: "insect resistant soybean plants, said soybean plants comprising SEQ ID NO: 14"); <i>id.</i> at 65:7-8 (Claim 5: "soybean plants comprising SEQ ID NO: 14"); July 10, 2013 Non-Final Rejection (Ex. R), at 6-8; October 10, 2013 Response to Office Action (Ex. S), at 5-6; November 5, 2013 Notice of Allowance (Ex. T), at 2-3.</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction ensures that the scope of the claims to soybean plants comprising SEQ ID NO 14, which uniquely defines an event DAS81419 soybean plant and is consistent with the breadth to which the claim was narrowed during prosecution.</p>

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>American Type Culture Collection under Accession No. PTA-12006.”)</p> <p>'363 patent, cl. 8 (“A seed or a part of the plant of claim 7, wherein said seed or part comprises SEQ ID NO: 14.”)</p> <p>'363 patent, cl. 9 (“A soybean plant, or part thereof, comprising the DNA sequence of SEQ ID NO: 14.”)</p> <p><i>E.g.</i>, '363 patent, 1:25-29 (“The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site.”), 1:35-38 (“For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events.”), 5:62-65 (“As alluded to above in the Background section, the introduction and integration of a transgene into a plant genome involves some random events (hence the name ‘event’ for a given insertion that is expressed).”), 7:51-54 (“This invention includes a method for producing an F<sub>1</sub> hybrid seed by crossing an exemplified plant with a different (e.g. in-bred parent)</p>	

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>plant and harvesting the resultant seed.”), 8:55-60 (“Methods of the subject invention include a method of producing an insect resistant/herbicide-tolerant soybean plant wherein said method comprises breeding with a plant of the subject invention.”), 8:64-9:1 (“For example, the subject invention can be used to track the subject event through breeding cycles with plants comprising other desirable traits, such as agronomic traits, disease tolerance or resistance, nematode tolerance or resistance and maturity date.”), 10:55-59 (“As used herein gene, event or trait ‘stacking’ is combining desired traits into one transgenic line. Plant breeders stack transgenic traits by making crosses between parents that each have a desired trait and then identifying offspring that have both of these desired traits.”)</p> <p>'363 patent, SEQ ID NO: 14 (“Sequence of soybean event 9582.814.19.1”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	



'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"first plant" (Unasserted Claim 6)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is first organism belonging to the kingdom Plantae</p> <p><b>Intrinsic Evidence:</b></p> <p>'363 patent, cl. 1 ("A method of controlling insects, said method comprising exposing insects to insect resistant soybean plants, said soybean plants comprising SEQ ID NO: 14 . . .")</p> <p>'363 patent, cl. 5 ("A method of controlling weeds in a soybean crop, said method comprising applying glufosinate herbicide to the soybean crop, said soybean crop comprising soybean plants comprising SEQ ID NO: 14.")</p> <p>'363 patent, cl. 6 ("A method of breeding a soybean plant, said method comprising: crossing a first plant with a second soybean plant to produce a third soybean plant, said first plant comprising DNA comprising SEQ ID NO: 14; and assaying said third soybean plant for the presence of SEQ ID NO: 14.")</p> <p>'363 patent, cl. 7 ("A soybean plant, wherein representative seed of said soybean plant has been deposited with the</p>	<p>An event DAS81419 soybean plant.</p> <p><b>Intrinsic Evidence:</b></p> <p>'363 Patent at 65:10-12 (Claim 6: "crossing a first plant with a second soybean plant to produce a third soybean plant, said first plant comprising DNA comprising SEQ ID NO: 14") July 10, 2013 Non-Final Rejection (Ex. R), at 6-8; October 10, 2013 Response to Office Action (Ex. S), at 5-6; November 5, 2013 Notice of Allowance (Ex. T), at 2-3.</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction logically follows the breeding method claimed and the identification of the first plant as comprising SEQ ID NO 14 and ensures that the scope of the claims is consistent with the breadth to which it was narrowed during prosecution.</p>

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>American Type Culture Collection under Accession No. PTA-12006.”)</p> <p>'363 patent, cl. 8 (“A seed or a part of the plant of claim 7, wherein said seed or part comprises SEQ ID NO: 14.”)</p> <p>'363 patent, cl. 9 (“A soybean plant, or part thereof, comprising the DNA sequence of SEQ ID NO: 14.”)</p> <p><i>E.g.</i>, '363 patent, 1:25-29 (“The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site.”), 1:35-38 (“For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events.”), 5:62-65 (“As alluded to above in the Background section, the introduction and integration of a transgene into a plant genome involves some random events (hence the name ‘event’ for a given insertion that is expressed).”), 7:51-54 (“This invention includes a method for producing an F<sub>1</sub> hybrid seed by crossing an exemplified plant with a different (e.g. in-bred parent)</p>	

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>plant and harvesting the resultant seed.”), 8:55-60 (“Methods of the subject invention include a method of producing an insect resistant/herbicide-tolerant soybean plant wherein said method comprises breeding with a plant of the subject invention.”), 8:64-9:1 (“For example, the subject invention can be used to track the subject event through breeding cycles with plants comprising other desirable traits, such as agronomic traits, disease tolerance or resistance, nematode tolerance or resistance and maturity date.”), 10:55-59 (“As used herein gene, event or trait ‘stacking’ is combining desired traits into one transgenic line. Plant breeders stack transgenic traits by making crosses between parents that each have a desired trait and then identifying offspring that have both of these desired traits.”)</p> <p>'363 patent, SEQ ID NO: 14 (“Sequence of soybean event 9582.814.19.1”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"plant" (Asserted Claims 7-9)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is organism belonging to the kingdom Plantae</p> <p><b>Intrinsic Evidence:</b></p> <p>'363 patent, cl. 1 ("A method of controlling insects, said method comprising exposing insects to insect resistant soybean plants, said soybean plants comprising SEQ ID NO: 14 . . .")</p> <p>'363 patent, cl. 5 ("A method of controlling weeds in a soybean crop, said method comprising applying glufosinate herbicide to the soybean crop, said soybean crop comprising soybean plants comprising SEQ ID NO: 14.")</p> <p>'363 patent, cl. 6 ("A method of breeding a soybean plant, said method comprising: crossing a first plant with a second soybean plant to produce a third soybean plant, said first plant comprising DNA comprising SEQ ID NO: 14; and assaying said third soybean plant for the presence of SEQ ID NO: 14.")</p> <p>'363 patent, cl. 7 ("A soybean plant, wherein representative seed of said soybean plant has been deposited with the</p>	<p>An event DAS81419 soybean plant.</p> <p><b>Intrinsic Evidence:</b></p> <p>'363 Patent at 65:14-16 (Claim 7: "A soybean plant, wherein representative seed of said soybean plant has been deposited with the American Type Culture Collection under Accession No. PTA-12006"); <i>id.</i> at 65:17-18 (Claim 8: "A seed or a part of the plant of claim 7, wherein said seed or part comprises SEQ ID NO: 14"); <i>id.</i> at 65:19-20 (Claim 9: "A soybean plant, or part thereof, comprising the DNA sequence of SEQ ID NO: 14") July 10, 2013 Non-Final Rejection (Ex. R), at 6-8; October 10, 2013 Response to Office Action (Ex. S), at 5-6; November 5, 2013 Notice of Allowance (Ex. T), at 2-3.</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction ensures that the scope of the claims is limited to soybean plants comprising SEQ ID NO 14, which uniquely defines an event DAS81419 soybean plant and is consistent with the breadth to which it was narrowed during prosecution.</p>

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>American Type Culture Collection under Accession No. PTA-12006.”)</p> <p>'363 patent, cl. 8 (“A seed or a part of the plant of claim 7, wherein said seed or part comprises SEQ ID NO: 14.”)</p> <p>'363 patent, cl. 9 (“A soybean plant, or part thereof, comprising the DNA sequence of SEQ ID NO: 14.”)</p> <p><i>E.g.</i>, '363 patent, 1:25-29 (“The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site.”), 1:35-38 (“For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events.”), 5:62-65 (“As alluded to above in the Background section, the introduction and integration of a transgene into a plant genome involves some random events (hence the name ‘event’ for a given insertion that is expressed).”), 7:51-54 (“This invention includes a method for producing an F<sub>1</sub> hybrid seed by crossing an exemplified plant with a different (e.g. in-bred parent)</p>	

'363 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>plant and harvesting the resultant seed.”), 8:55-60 (“Methods of the subject invention include a method of producing an insect resistant/herbicide-tolerant soybean plant wherein said method comprises breeding with a plant of the subject invention.”), 8:64-9:1 (“For example, the subject invention can be used to track the subject event through breeding cycles with plants comprising other desirable traits, such as agronomic traits, disease tolerance or resistance, nematode tolerance or resistance and maturity date.”), 10:55-59 (“As used herein gene, event or trait ‘stacking’ is combining desired traits into one transgenic line. Plant breeders stack transgenic traits by making crosses between parents that each have a desired trait and then identifying offspring that have both of these desired traits.”)</p> <p>'363 patent, SEQ ID NO: 14 (“Sequence of soybean event 9582.814.19.1”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	

**E. '441 Patent, (Ex. F)**

'441 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"seed" (Asserted Claim 2)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is ripened ovule of a flowering plant that may develop into a new plant</p> <p><b>Intrinsic Evidence:</b></p> <p>'441 patent, cl. 2 ("A soybean plant, seed, or other part of said plant comprising the polynucleotide of claim 1.")</p> <p>'441 patent, 4:17-21 ("The invention also includes soybean plant cells and plant parts including, but are not limited to pollen, ovule, flowers, shoots, roots, and leaves, and nuclei of vegetative cells, pollen cells, seed and seed meal, and egg cells, that contain soybean event 9852.814.19.1")</p> <p>'441 patent, 7:38-45 ("The subject invention also includes a herbicide-tolerant soybean plant grown from a seed deposited with the ATCC Deposit No. as described herein. The subject invention further includes parts of said plant, such as leaves, tissue samples, seeds produced by said plant, pollen, and the like . . .")</p> <p>'441 patent, 7:66-8:2 ("An insect resistant/glufosinate-tolerant soybean plant of the subject invention can be bred by first</p>	<p>Plain and ordinary meaning, which is a seed coat, food store, and plant embryo.</p> <p><b>Intrinsic Evidence:</b></p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction confirms that the term retains its plain and ordinary meaning to a person of ordinary skill in the art. The plain and ordinary meaning helps clarify that the seed already contains a plant in embryo form rather than something that may become a plant. Construing this term is relevant to the infringement claim under 35 U.S.C. § 271(f)(2).</p>

'441 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>sexually crossing a first parental soybean plant consisting of a soybean plant grown from seed of any one of the lines referred to herein . . .")</p> <p>U.S. Patent Application No. 13/559,177 Prosecution History, July 10, 2013 Non-Final Office Action (Ex. R) at 17 ("Claim 9 is directed to the seed of the soybean plant of claim 8. Because the claims do not required [sic] that the seed be grown from the deposited seeds and comprise the event, the term 'seeds' is given the broadest reasonable interpretation as encompassing the seeds obtained from the plant of any filial generation of the plants grown from the deposited seeds.")</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	
<p>"plant" (Asserted Claim 2)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is organism belonging to the kingdom Plantae</p> <p><b>Intrinsic Evidence:</b></p>	<p>An event DAS81419 soybean plant.</p> <p><b>Intrinsic Evidence:</b></p> <p>'441 Patent at 10:53-62 ("The subject invention discloses herein a specific site on chromosome 02 in the soybean genome that is excellent for insertion of</p>



'441 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>'441 patent, cl. 2 ("A soybean plant, seed, or other part of said plant comprising the polynucleotide of claim 1.")</p> <p><i>E.g.</i>, '441 patent, 1:27-31 ("The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site."), 1:37-40 ("For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events."), 6:1-4 ("As alluded to above in the Background section, the introduction and integration of a transgene into a plant genome involves some random events (hence the name 'event' for a given insertion that is expressed)."), 7:58-62 ("This invention includes a method for producing an F<sub>1</sub> hybrid seed by crossing an exemplified plant with a different (e.g. in-bred parent) plant and harvesting the resultant hybrid seed."), 8:64-67 ("Methods of the subject invention include a method of producing an insect resistant/herbicide-tolerant soybean plant wherein said method comprises breeding with a plant of the subject invention."), 9:6-10 ("For example,</p>	<p>heterologous nucleic acids. Thus, the subject invention provides methods to introduce heterologous nucleic acids of interest into this pre-established target site or in the vicinity of this target site. The subject invention also encompasses a soybean seed and/or a soybean plant comprising any heterologous nucleotide sequence inserted at the disclosed target site or in the general vicinity of such site."); <i>id.</i> at 8:35-37 ("Likewise an insect resistant/glufosinate-tolerant soybean plant of the subject invention can be transformed with additional transgenes using methods known in the art"); <i>id.</i> at 11:47-57 ("A preferred plant, or a seed, of the subject invention comprises in its genome operative cry1F v3, cry1Ac synpro and pat v6 nucleotide sequences, as identified herein, together with at least 20-500 or more contiguous flanking nucleotides on both sides of the insert, as identified herein. Unless indicated otherwise, reference to flanking sequences refers to those identified with respect to SEQ ID NOS: 1 and 2. All or part of these flanking sequences could be expected to be transferred to progeny that receives the inserted DNA as a result of a sexual cross of a parental line that includes the event."); September 8, 2016 Non-Final Rejection (Ex. X), at 2; December 8, 2016 Response</p>

'441 Patent Term (Asserted Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>the subject invention can be used to track the subject event through breeding cycles with plants comprising other desirable traits, such as agronomic traits, disease tolerance or resistance, nematode tolerance or resistance and maturity date.”), 11:1-5 (“As used herein gene, event or trait ‘stacking’ is combining desired traits into one transgenic line. Plant breeders stack transgenic traits by making crosses between parents that each have a desired trait and then identifying offspring that have both of these desired traits.”)</p> <p>'441 patent, SEQ ID NO: 14 (“Sequence of soybean event 9582.814.19.1”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	<p>to Non-Final Office Action (Ex. Y) at 7; March 7, 2017 Notice of Allowance (Ex. Z) at 3.</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>The proposed construction ensures that the scope of the claims to soybean plants comprising SEQ ID NO 14, which uniquely defines an event DAS81419 soybean plant and is consistent with the breadth to which it was narrowed during prosecution.</p>

**F. '378 Patent, (Ex. G)**

'378 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
<p>"flanked by" (Unasserted Claim 1)</p> <p><b>Identified by Defendants</b></p>	<p>Plain and ordinary meaning, which is joined or connected at the side to</p> <p><b>Intrinsic Evidence:</b></p> <p>'378 patent, cl. 1 ("A corn plant comprising a DNA construct, said DNA construct comprising a first and a second expression cassette, wherein said first expression cassette in operable linkage comprises (a) a maize ubiquitin promoter; (b) a 5' untranslated exon of a maize ubiquitin gene; (c) a maize ubiquitin intron; (d) a Cry1F encoding DNA molecule; and (e) a 3' ORF25 transcriptional terminator; and said second expression cassette comprising in operable linkage (i) a CaMV 35S promoter; (ii) a pat encoding DNA molecule; and (iii) a 3' transcriptional terminator from (CaMV) 35 S comprising the nucleic acid sequence of positions 5843 to 6032 of SEQ ID NO: 25; and wherein the first and second expression cassettes are flanked by SEQ ID NO: 26 at the 5' end and SEQ ID NO: 27 at the 3' end.</p> <p>'378 patent, Fig. 1</p> <p>'378 patent, 15:62-67 ("Alternatively, primer pairs can be derived from flanking</p>	<p>Adjacent to.</p> <p><b>Intrinsic Evidence:</b></p> <p>'378 Patent at 2:32-36 ("[polymerase chain reaction or DNA hybridization using nucleic acid probes] may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known"); <i>id.</i> at 7:26-35 ("A 'flanking region' or 'flanking sequence' as used herein refers to a sequence of at least 20 base pair, preferably at least 50 base pair, and up to 5000 base pair which is located either immediately upstream of and contiguous with or immediately downstream of and contiguous with the original foreign insert DNA molecule. Transformation procedures leading to random integration of the foreign DNA will result in transformants containing different flanking regions characteristic and unique for each transformant."); <i>id.</i> at 9:49-58 ("The term 'event' also refers to DNA from the original transformant comprising the inserted DNA and flanking sequence immediately adjacent to the</p>

'378 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence of the PHI8999A expression construct, see FIG. 1, approximately 6.2 Kb in size.”)</p> <p>'378 patent, 2:32-36 (“As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known.”)</p> <p>'378 patent, 7:40-45 (“A ‘junction’ is a point where 2 specific DNA fragments join. For example, a junction exists where insert DNA joins flanking DNA. A junction point also exists in a transformed organism where 2 DNA fragments join together in a manner that is modified from that found in the native organism.”)</p> <p>'378 patent, 9:49-58 (“The term ‘event’ also refers to DNA from the original transformant comprising the inserted DNA and flanking sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the</p>	<p>inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA”): <i>id.</i> at 16:24-36 (“The amplicon produced by these methods may be detected by a plurality of techniques, including, but not limited to, Genetic Bit Analysis (Nikiforov, et al. Nucleic Acid Res. 22:4167-4175, 1994) where a DNA oligonucleotide is designed which overlaps both the adjacent flanking DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking sequence) a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base”).</p> <p><b>Why Resolution Makes a Difference:</b></p>

'378 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.”)</p> <p>'378 patent, 15:47-57 (“For example, to determine whether a corn plant resulting from a sexual cross contains transgenic event genomic DNA from the corn plant of the present invention, DNA extracted from the corn plant tissue sample may be subjected to a nucleic acid amplification method using a DNA primer pair that includes a first primer derived from flanking sequence adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA.”)</p> <p>'378 patent, 16:24-36 (“The amplicon produced by these methods may be detected by a plurality of techniques, including, but not limited to, Genetic Bit Analysis . . . where a DNA oligonucleotide is designed which overlaps both the adjacent flanking DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of</p>	<p>The proposed construction confirms that the claimed plant is a TC1507 event plant by requiring the two flanking sequences adjacent to the first and second expression cassette located at positions unique to TC1507 event. Allowing the flanking sequences to be joined to the expression cassettes with any number of base pairs in between would impermissibly broaden the scope of the claim to cover possibilities that are not enabled by the specification.</p>

'378 Patent Term (Claims)	Corteva's Proposed Construction	Inari's Proposed Construction
	<p>a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking sequence) a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base.”)</p> <p><b>Why Resolution Makes a Difference:</b></p> <p>Corteva believes that this term does not require construction, but if it does, Corteva's construction should prevail.</p>	

# **Exhibit B**

(12) **United States Patent**  
**Diehn et al.**

(10) **Patent No.:** **US 8,575,434 B2**  
(45) **Date of Patent:** **Nov. 5, 2013**

(54) **MAIZE EVENT DP-004114-3 AND METHODS FOR DETECTION THEREOF**

FOREIGN PATENT DOCUMENTS

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(73) Assignees: **Pioneer Hi Bred International Inc.**, Johnston, IA (US); **E I du Pont de Nemours and Company**, Wilmington, DE (US)

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(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 210 days.

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(21) Appl. No.: **12/970,052**

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(22) Filed: **Dec. 16, 2010**

Storer, et al., "Field Measures of Western Corn Rootworm (Coleoptera: Chrysomelidae) Mortality Caused by Cry34/35Ab1 Proteins Expressed in Maize Event 59122 and Implications for Trait Durability." *J. Econ. Entomology*, vol. 99(4):1381-1387 (2006).

(65) **Prior Publication Data**

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Moellenbeck, et al., "Insecticidal proteins from *Bacillus thuringiensis* protect corn from corn rootworms." *Nature Biotechnology*, vol. 19: 668-672 (2001).

(51) **Int. Cl.**  
**A23L 1/28** (2006.01)  
**A01H 5/00** (2006.01)  
**A01H 5/10** (2006.01)  
**A01H 1/00** (2006.01)  
**C07H 21/04** (2006.01)

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(52) **U.S. Cl.**  
USPC ..... **800/302**; 800/260; 800/279; 800/320.1;  
800/265; 800/275; 536/23.71; 426/655; 426/615

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(58) **Field of Classification Search**  
None  
See application file for complete search history.

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*Primary Examiner* — Anne Kubelik  
*Assistant Examiner* — Jeffrey Bolland

(57) **ABSTRACT**

The invention provides DNA compositions that relate to transgenic insect resistant maize plants. Also provided are assays for detecting the presence of the maize DP-004114-3 event based on the DNA sequence of the recombinant construct inserted into the maize genome and the DNA sequences flanking the insertion site. Kits and conditions useful in conducting the assays are provided.

**28 Claims, 5 Drawing Sheets**



U.S. Patent

Nov. 5, 2013

Sheet 1 of 5

US 8,575,434 B2

Figure 1

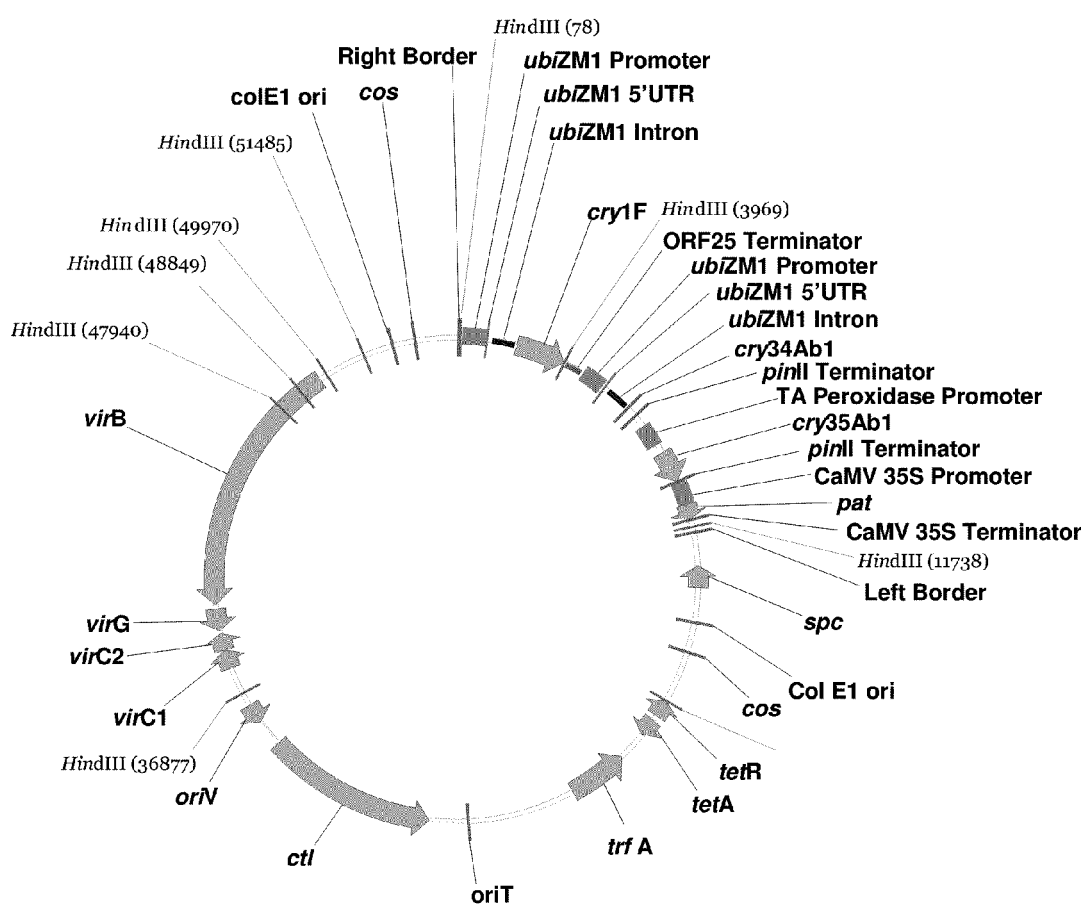


Figure 2

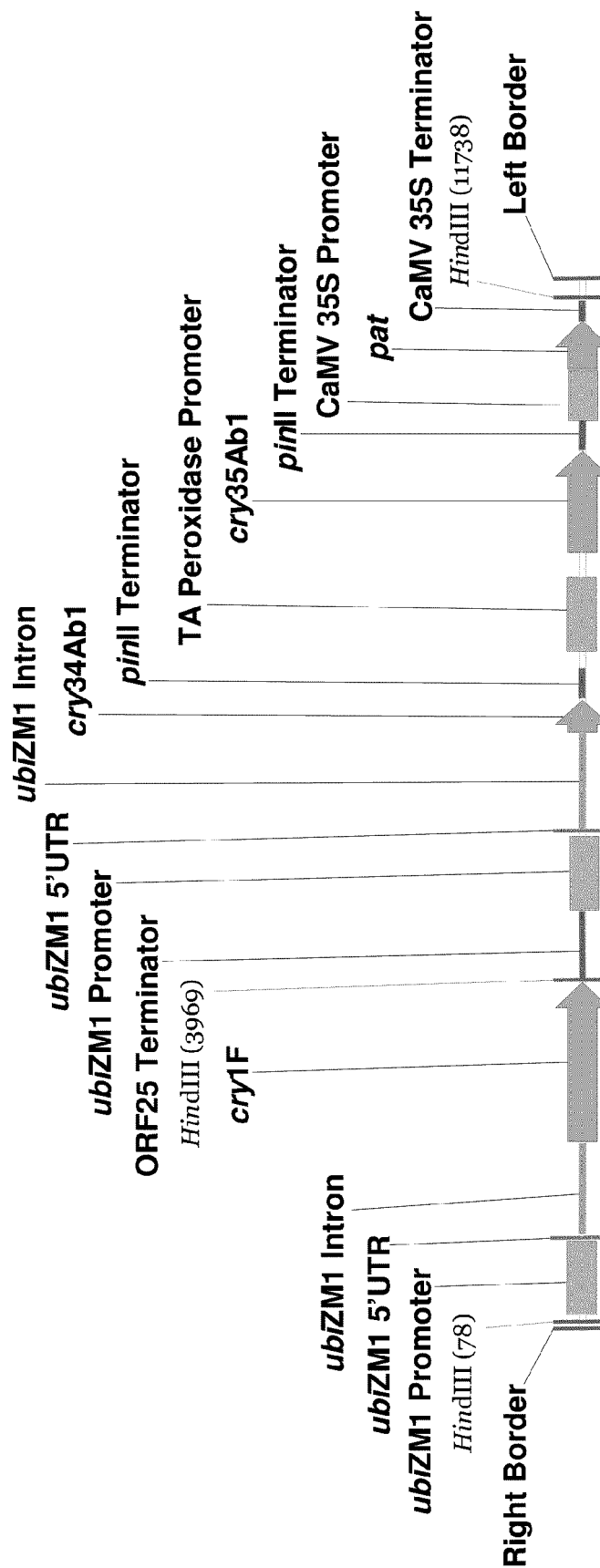
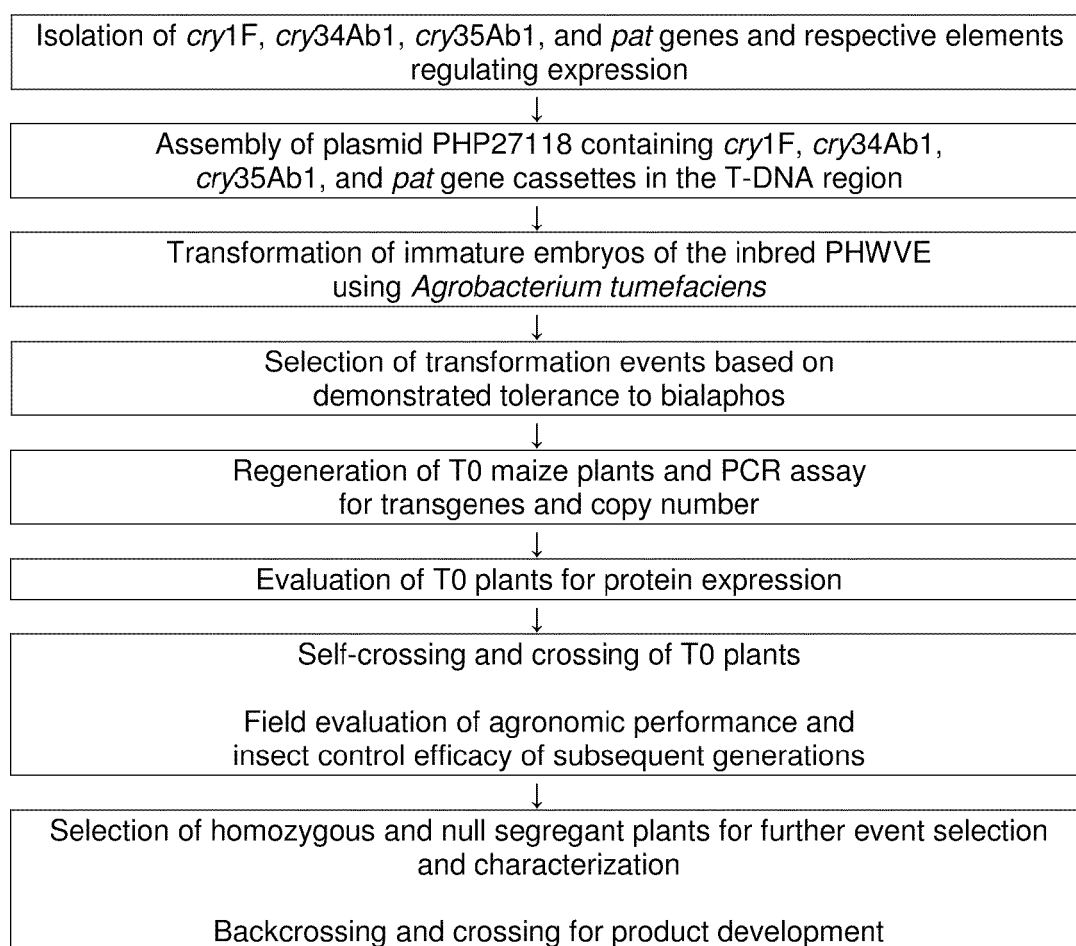


Figure 3



U.S. Patent

Nov. 5, 2013

Sheet 4 of 5

US 8,575,434 B2

Figure 4

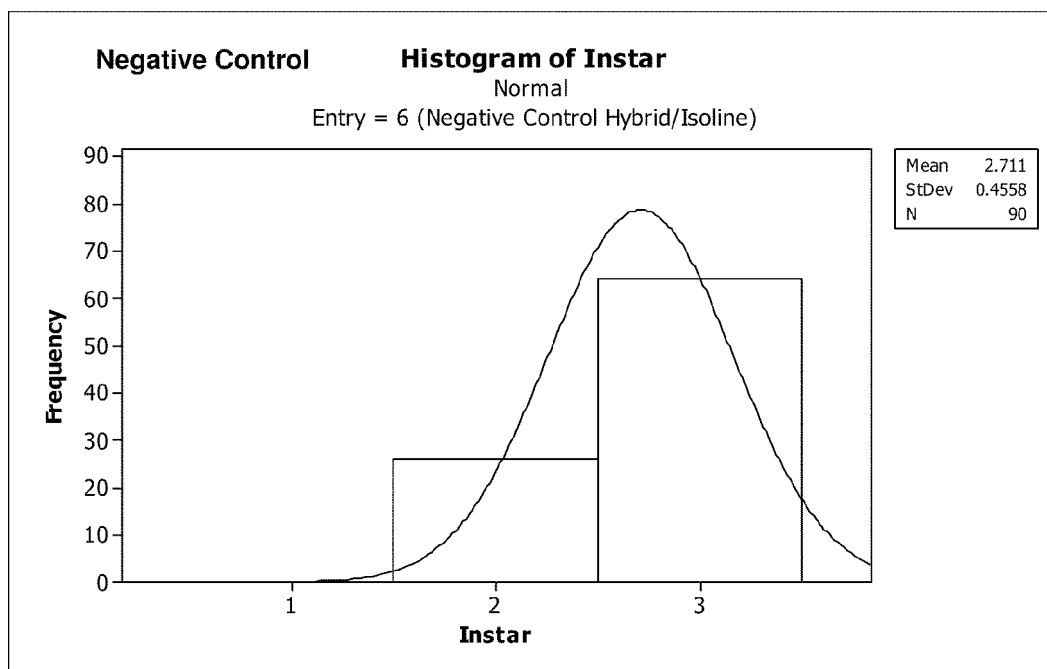
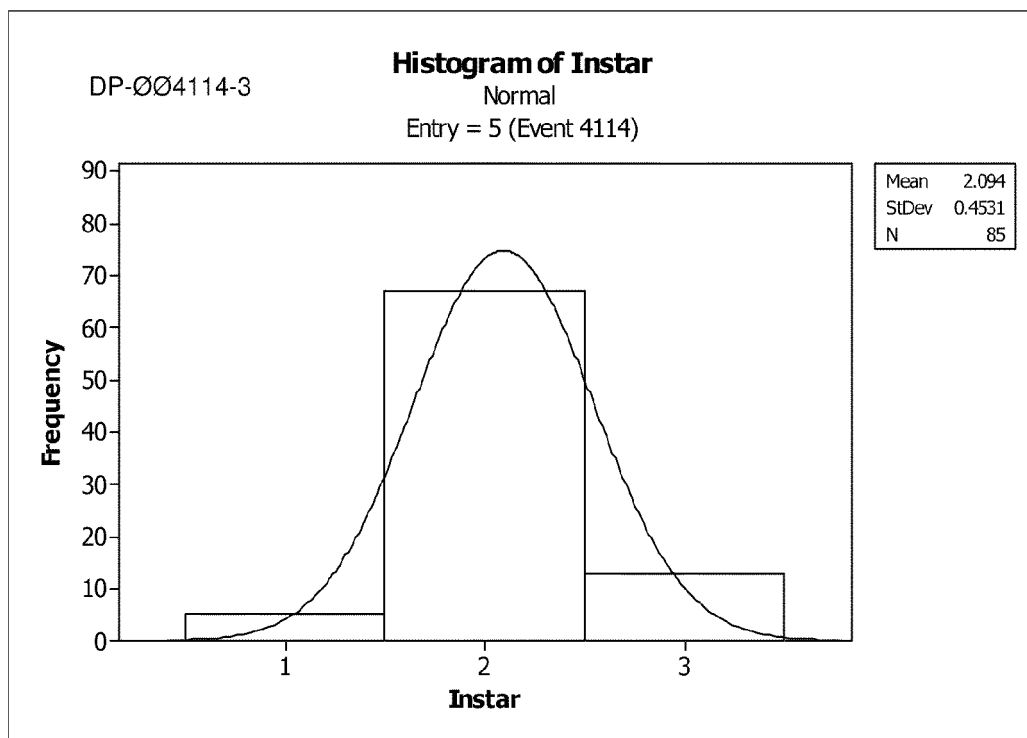
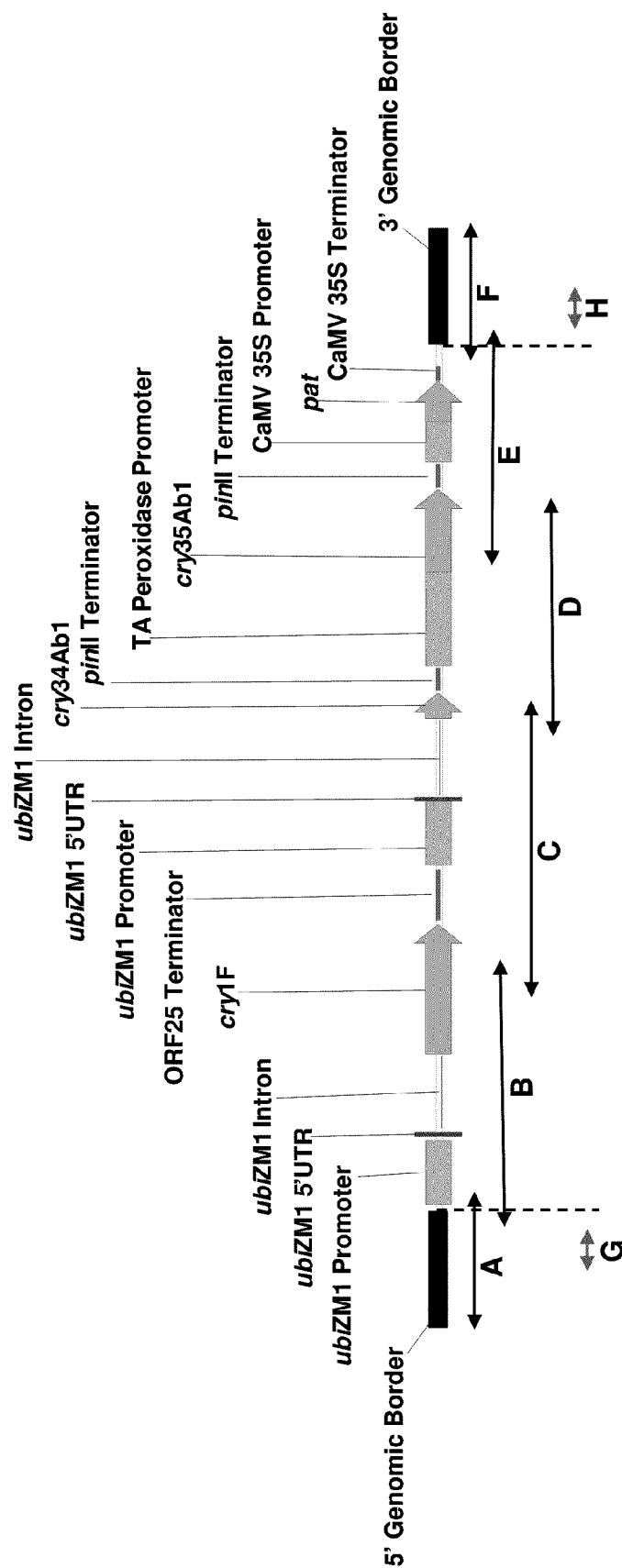


Figure 5



US 8,575,434 B2

1

**MAIZE EVENT DP-004114-3 AND METHODS  
FOR DETECTION THEREOF****CROSS-REFERENCE TO RELATED  
APPLICATIONS**

This application claims the benefit of U.S. Provisional Application No. 61/413,536, filed on Nov. 15, 2010 and U.S. Provisional Application No. 61/287,462, filed Dec. 17, 2009, the contents of which are herein incorporated by reference in their entirety.

**FIELD OF INVENTION**

Embodiments of the present invention relate to the field of plant molecular biology, specifically embodiment of the invention relate to DNA constructs for conferring insect resistance to a plant. Embodiments of the invention more specifically relate to insect resistant corn plant event DP-004114-3 and to assays for detecting the presence of corn event DP-004114-3 in a sample and compositions thereof.

**BACKGROUND OF INVENTION**

An embodiment of this invention relates to the insect resistant corn (*Zea mays*) plant DP-004114-3, also referred to as "maize line DP-004114-3," "maize event DP-004114-3," and "4114 maize," and to the DNA plant expression construct of corn plant DP-004114-3 and the detection of the transgene/flanking insertion region in corn plant DP-004114-3 and progeny thereof.

Corn is an important crop and is a primary food source in many areas of the world. Damage caused by insect pests is a major factor in the loss of the world's corn crops, despite the use of protective measures such as chemical pesticides. In view of this, insect resistance has been genetically engineered into crops such as corn in order to control insect damage and to reduce the need for traditional chemical pesticides. One group of genes which have been utilized for the production of transgenic insect resistant crops is the delta-endotoxin group from *Bacillus thuringiensis* (Bt). Delta-endotoxins have been successfully expressed in crop plants such as cotton, potatoes, rice, sunflower, as well as corn, and have proven to provide excellent control over insect pests. (Perlak, F. J. et al. (1990) *Bio/Technology* 8:939-943; Perlak, F. J. et al. (1993) *Plant Mol. Biol.* 22:313-321; Fujimoto, H. et al. (1993) *Bio/Technology* 11:1151-1155; Tu et al. (2000) *Nature Biotechnology* 18:1101-1104; PCT publication WO 01/13731; and Bing, J. W. et al. (2000) Efficacy of Cry1F Transgenic Maize, 14<sup>th</sup> Biennial International Plant Resistance to Insects Workshop, Fort Collins, Colo.).

The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site (Weising et al. (1988) *Ann. Rev. Genet.* 22:421-477). At the same time the presence of the transgene at different locations in the genome will influence the overall phenotype of the plant in different ways. For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of an introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant

2

tissues, that may not correspond to the patterns expected from transcriptional regulatory elements present in the introduced gene construct. For this reason, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual outcrossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure reliable gene expression in a number of varieties that are well adapted to local growing conditions.

It would be advantageous to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene of interest. In addition, a method for detecting a particular event would be helpful for complying with regulations requiring the pre-market approval and labeling of foods derived from recombinant crop plants, for example, or for use in environmental monitoring, monitoring traits in crops in the field, or monitoring products derived from a crop harvest, as well as for use in ensuring compliance of parties subject to regulatory or contractual terms.

It is possible to detect the presence of a transgene by any nucleic acid detection method known in the art including, but not limited to, the polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc., because for many DNA constructs, the coding region is interchangeable. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known. For example, an event-specific PCR assay is described in U.S. Pat. No. 6,395,485 for the detection of elite event GAT-ZM1. Accordingly, it would be desirable to have a simple and discriminative method for the identification of event DP-004114-3.

**SUMMARY OF INVENTION**

Embodiments of this invention relate to methods for producing and selecting an insect resistant monocot crop plant. More specifically, a DNA construct is provided that when expressed in plant cells and plants confers resistance to insects. According to one aspect of the invention, a DNA construct, capable of introduction into and replication in a host cell, is provided that when expressed in plant cells and plants confers insect resistance to the plant cells and plants. Maize event DP-004114-3 was produced by *Agrobacterium*-mediated transformation with plasmid PHP27118. This event contains the cry1F, cry34Ab1, cry35Ab1, and pat gene cassettes, which confer resistance to certain lepidopteran and coleopteran pests, as well as tolerance to phosphinothricin.

Specifically, the first cassette contains a truncated version of the cry1F gene from *Bacillus thuringiensis* var. *aizawai*. The insertion of the cry1F gene confers resistance to damage by lepidopteran pests. The Cry1F protein (SEQ ID NO: 1) is comprised of 605 amino acids and has a molecular weight of approximately 68 kDa. The expression of the cry1F gene is controlled by the maize polyubiquitin promoter (Christensen et al. (1992) *Plant Mol. Biol.* 118(4):675-89), providing constitutive expression of the Cry1F protein in maize. This region also includes the 5' untranslated region (UTR) and intron associated with the native polyubiquitin promoter. The term-

US 8,575,434 B2

3

nator for the cry1F gene is the poly(A) addition signal from Open Reading Frame 25 (ORF 25) of the *Agrobacterium tumefaciens* Ti plasmid pTi15955 (Barker et al. (1983) *Plant Mol. Biol.* 2:335-350).

The second cassette contains the cry34Ab1 gene isolated from *Bacillus thuringiensis* strain PS149B1 (U.S. Pat. Nos. 6,127,180; 6,624,145 and 6,340,593). The Cry34Ab1 protein (SEQ ID NO: 2) is 123 amino acid residues in length and has a molecular weight of approximately 14 kDa. The expression of the cry34Ab1 gene is controlled by a second copy of the maize polyubiquitin promoter with 5' UTR and intron (Christensen et al., 1992, supra). The terminator for the cry34Ab1 gene is the pinII terminator (Keil et al. (1986) *Nucleic Acids Res.* 14:5641-5650; An et al. (1989) *Plant Cell* 1:115-22).

The third gene cassette contains the cry35Ab1 gene, also isolated from *Bacillus thuringiensis* strain PS149B1 (U.S. Pat. Nos. 6,083,499; 6,548,291 and 6,340,593). The Cry35Ab1 protein (SEQ ID NO: 3) has a length of 383 amino acids and a molecular weight of approximately 44 kDa. Simultaneous expression of the Cry34Ab1 and Cry35Ab1 proteins in the plant confers resistance to coleopteran insects. The expression of the cry35Ab1 gene is controlled by the *Triticum aestivum* (wheat) peroxidase promoter and leader sequence (Hertig et al. (1991) *Plant Mol. Biol.* 16:171-174). The terminator for the cry35Ab1 gene is a second copy of the pinII terminator (Keil et al., 1986, supra; An et al., 1989, supra).

The fourth and final gene cassette contains a version of the phosphinothricin acetyl transferase gene from *Streptomyces viridochromogenes* (pat) that has been optimized for expression in maize. The pat gene expresses the phosphinothricin acetyl transferase enzyme (PAT) that confers tolerance to phosphinothricin. The PAT protein (SEQ ID NO: 4) is 183 amino acids residues in length and has a molecular weight of approximately 21 kDa. Expression of the pat gene is controlled by the promoter and terminator regions from the CaMV 35S transcript (Franck et al. (1980) *Cell* 21:285-294; Odell et al. (1985) *Nature* 313:810-812; Pietrzak, et al. (1986) *Nucleic Acids Res.* 14(14):5857-5868). Plants containing the DNA constructs are also provided.

According to another embodiment of the invention, compositions and methods are provided for identifying a novel corn plant designated DP-004114-3. The methods are based on primers or probes which specifically recognize the 5' and/or 3' flanking sequence of DP-004114-3. DNA molecules are provided that comprise primer sequences that when utilized in a PCR reaction will produce amplicons unique to the transgenic event DP-004114-3. The corn plant and seed comprising these molecules is an embodiment of this invention. Further, kits utilizing these primer sequences for the identification of the DP-004114-3 event are provided.

An additional embodiment of the invention relates to the specific flanking sequence of DP-004114-3 described herein, which can be used to develop specific identification methods for DP-004114-3 in biological samples. More particularly, the invention relates to the 5' and/or 3' flanking regions of DP-004114-3 which can be used for the development of specific primers and probes. A further embodiment of the invention relates to identification methods for the presence of DP-004114-3 in biological samples based on the use of such specific primers or probes.

According to another embodiment of the invention, methods of detecting the presence of DNA corresponding to the corn event DP-004114-3 in a sample are provided. Such methods comprise: (a) contacting the sample comprising DNA with a DNA primer set, that when used in a nucleic acid amplification reaction with genomic DNA extracted from

4

corn event DP-004114-3 produces an amplicon that is diagnostic for corn event DP-004114-3; (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon.

According to another embodiment of the invention, methods of detecting the presence of a DNA molecule corresponding to the DP-004114-3 event in a sample, such methods comprising: (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that hybridizes under stringent hybridization conditions with DNA extracted from corn event DP-004114-3 and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA. More specifically, a method for detecting the presence of a DNA molecule corresponding to the DP-004114-3 event in a sample, such methods, consisting of (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that consists of sequences that are unique to the event, e.g. junction sequences, wherein said DNA probe molecule hybridizes under stringent hybridization conditions with DNA extracted from corn event DP-004114-3 and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.

In addition, a kit and methods for identifying event DP-004114-3 in a biological sample which detects a DP-004114-3 specific region are provided.

DNA molecules are provided that comprise at least one junction sequence of DP-004114-3; wherein a junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the corn cell flanking the insertion site, i.e. flanking DNA, and is diagnostic for the DP-004114-3 event.

According to another embodiment of the invention, methods of producing an insect resistant corn plant that comprise the steps of: (a) sexually crossing a first parental corn line comprising the expression cassettes of the invention, which confers resistance to insects, and a second parental corn line that lacks insect resistance, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that is insect resistant. Such methods may optionally comprise the further step of back-crossing the progeny plant to the second parental corn line to producing a true-breeding corn plant that is insect resistant.

A further embodiment of the invention provides a method of producing a corn plant that is resistant to insects comprising transforming a corn cell with the DNA construct PHP27118, growing the transformed corn cell into a corn plant, selecting the corn plant that shows resistance to insects, and further growing the corn plant into a fertile corn plant. The fertile corn plant can be self pollinated or crossed with compatible corn varieties to produce insect resistant progeny.

Another embodiment of the invention further relates to a DNA detection kit for identifying maize event DP-004114-3 in biological samples. The kit comprises a first primer which specifically recognizes the 5' or 3' flanking region of DP-004114-3, and a second primer which specifically recognizes a sequence within the foreign DNA of DP-004114-3, or within the flanking DNA, for use in a PCR identification protocol. A further embodiment of the invention relates to a kit for identifying event DP-004114-3 in biological samples, which kit comprises a specific probe having a sequence which corresponds or is complementary to, a sequence having between 80% and 100% sequence identity with a specific



US 8,575,434 B2

5

region of event DP-004114-3. The sequence of the probe corresponds to a specific region comprising part of the 5' or 3' flanking region of event DP-004114-3.

The methods and kits encompassed by the embodiments of the present invention can be used for different purposes such as, but not limited to the following: to identify event DP-004114-3 in plants, plant material or in products such as, but not limited to, food or feed products (fresh or processed) comprising, or derived from plant material; additionally or alternatively, the methods and kits can be used to identify transgenic plant material for purposes of segregation between transgenic and non-transgenic material; additionally or alternatively, the methods and kits can be used to determine the quality of plant material comprising maize event DP-004114-3. The kits may also contain the reagents and materials necessary for the performance of the detection method.

A further embodiment of this invention relates to the DP-004114-3 corn plant or its parts, including, but not limited to, pollen, ovules, vegetative cells, the nuclei of pollen cells, and the nuclei of egg cells of the corn plant DP-004114-3 and the progeny derived thereof. The corn plant and seed of DP-004114-3 from which the DNA primer molecules provide a specific amplicon product is an embodiment of the invention.

The foregoing and other aspects of the invention will become more apparent from the following detailed description and accompanying drawing.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Schematic diagram of plasmid PHP27118 with genetic elements indicated and Hind III restriction enzyme sites. Plasmid size is 54910 bp.

FIG. 2. Schematic diagram of the T-DNA indicating the cry1F, cry34Ab1, cry35Ab1, and pat genes (arrows) along with their respective regulatory elements. Hind III restriction enzyme sites within the T-DNA are indicated. The size of the T-DNA is 11978 bp.

FIG. 3. Schematic Diagram of the Transformation and Development of DP-004114-3.

FIG. 4. Western corn rootworm (WCRW) larvae developmental effects in the sub-lethal seedling assay employing maize hybrid seedlings in the same genetic background: DP-004114-3 maize with an isolate as a negative control. Results are based on three replicates. Graphic profiles show the percent of larvae in each of three instars at 17 days post egg hatch. A shift towards instar 3 indicates a decrease in efficacy.

FIG. 5. Schematic representation of the insert and genomic border regions sequenced in 4114 maize. The diagram indicates the PCR fragments generated from 4114 maize genomic DNA that were cloned and sequenced: fragments A through F. The vertical dash line represents the genomic border/insert junctions. Fragment G and H represent the 5' and 3' genomic border regions, respectively. Figure is not drawn to scale.

#### DETAILED DESCRIPTION

The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger et al., *Glossary of Genetics: Classical and Molecular*, 5<sup>th</sup> edition, Springer-Verlag; New York, 1991;

6

and Lewin, *Genes V*, Oxford University Press: New York, 1994. The nomenclature for DNA bases as set forth at 37 CFR §1.822 is used.

The following table sets forth abbreviations used throughout this document, and in particular in the Examples section.

Table of Abbreviations

4114 maize	Maize containing event DP-004114-3
bp	Base pair
Bt	<i>Bacillus thuringiensis</i>
CaMV	Cauliflower mosaic virus
cry1F	cry1F gene from <i>Bacillus thuringiensis</i> var. <i>aizawai</i>
Cry1F	Protein from cry1F gene
cry34Ab1	cry34Ab1 gene from <i>Bacillus thuringiensis</i> strain PS149B1
Cry34Ab1	Protein from cry34Ab1
cry35Ab1	cry35Ab1 gene from <i>Bacillus thuringiensis</i> strain PS149B1
Cry35Ab1	Protein from cry35Ab1 gene
kb	Kilobase pair
kDa	KiloDalton
LB	Left T-DNA border
pat	phosphinothricin acetyl transferase gene
PAT	Protein from phosphinothricin acetyl transferase gene
PCR	Polymerase chain reaction
pinII	Proteinase inhibitor II gene from <i>Solanum tuberosum</i>
RB	Right T-DNA border
T-DNA	The transfer DNA portion of the <i>Agrobacterium</i> transformation plasmid between the Left and Right Borders that is expected to be transferred to the plant genome
UTR	Untranslated region
ECB	European corn borer ( <i>Ostrinia nubilalis</i> )
FAW	Fall armyworm ( <i>Spodoptera frugiperda</i> )
WCRW	western corn rootworm ( <i>Diabrotica virgifera virgifera</i> )

Compositions of this disclosure include seed deposited as Patent Deposit No. PTA-11506 and plants, plant cells, and seed derived therefrom. Applicant(s) have made a deposit of at least 2500 seeds of maize event DP-004114-3 with the American Type Culture Collection (ATCC), Manassas, Va. 20110-2209 USA, on Nov. 24, 2010 and the deposits were assigned ATCC Deposit No. PTA-11506. These deposits will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. These deposits were made merely as a convenience for those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The seeds deposited with the ATCC on Nov. 24, 2010 were taken from the deposit maintained by Pioneer Hi-Bred International, Inc., 7250 NW 62<sup>nd</sup> Avenue, Johnston, Iowa 50131-1000. Access to this deposit will be available during the pendency of the application to the Commissioner of Patents and Trademarks and persons determined by the Commissioner to be entitled thereto upon request. Upon allowance of any claims in the application, the Applicant(s) will make available to the public, pursuant to 37 C.F.R. §1.808, sample(s) of the deposit of at least 2500 seeds of hybrid maize with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va. 20110-2209. This deposit of seed of maize event DP-004114-3 will be maintained in the ATCC depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the enforceable life of the patent, whichever is longer, and will be replaced if it becomes nonviable during that period. Additionally, Applicant(s) have satisfied all the requirements of 37 C.F.R. §§1.801-1.809, including providing an indication of the viability of the sample upon deposit. Applicant(s) have no authority to waive any restrictions



US 8,575,434 B2

7

imposed by law on the transfer of biological material or its transportation in commerce. Applicant(s) do not waive any infringement of their rights granted under this patent or rights applicable to event DP-004114-3 under the Plant Variety Protection Act (7 USC 2321 et seq.). Unauthorized seed multiplication prohibited. The seed may be regulated.

As used herein, the term “comprising” means “including but not limited to.”

As used herein, the term “corn” means *Zea mays* or maize and includes all plant varieties that can be bred with corn, including wild maize species.

As used herein, the term “DP-004114-3 specific” refers to a nucleotide sequence which is suitable for discriminatively identifying event DP-004114-3 in plants, plant material, or in products such as, but not limited to, food or feed products (fresh or processed) comprising, or derived from plant material.

As used herein, the terms “insect resistant” and “impacting insect pests” refers to effecting changes in insect feeding, growth, and/or behavior at any stage of development, including but not limited to: killing the insect; retarding growth; preventing reproductive capability; inhibiting feeding; and the like.

As used herein, the terms “pesticidal activity” and “insecticidal activity” are used synonymously to refer to activity of an organism or a substance (such as, for example, a protein) that can be measured by numerous parameters including, but not limited to, pest mortality, pest weight loss, pest attraction, pest repellency, and other behavioral and physical changes of a pest after feeding on and/or exposure to the organism or substance for an appropriate length of time. For example “pesticidal proteins” are proteins that display pesticidal activity by themselves or in combination with other proteins.

“Coding sequence” refers to a nucleotide sequence that codes for a specific amino acid sequence. As used herein, the terms “encoding” or “encoded” when used in the context of a specified nucleic acid mean that the nucleic acid comprises the requisite information to guide translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid or may lack such intervening non-translated sequences (e.g., as in cDNA).

“Gene” refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. “Native gene” refers to a gene as found in nature with its own regulatory sequences. “Chimeric gene” refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. “Endogenous gene” refers to a native gene in its natural location in the genome of an organism. “Foreign” refers to material not normally found in the location of interest. Thus “foreign DNA” may comprise both recombinant DNA as well as newly introduced, rearranged DNA of the plant. A “foreign” gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation

8

procedure. The site in the plant genome where a recombinant DNA has been inserted may be referred to as the “insertion site” or “target site”.

As used herein, “insert DNA” refers to the heterologous DNA within the expression cassettes used to transform the plant material while “flanking DNA” can exist of either genomic DNA naturally present in an organism such as a plant, or foreign (heterologous) DNA introduced via the transformation process which is extraneous to the original insert DNA molecule, e.g. fragments associated with the transformation event. A “flanking region” or “flanking sequence” as used herein refers to a sequence of at least 20 bp, preferably at least 50 bp, and up to 5000 bp, which is located either immediately upstream of and contiguous with or immediately downstream of and contiguous with the original foreign insert DNA molecule. Transformation procedures leading to random integration of the foreign DNA will result in transformants containing different flanking regions characteristic and unique for each transformant. When recombinant DNA is introduced into a plant through traditional crossing, its flanking regions will generally not be changed. Transformants will also contain unique junctions between a piece of heterologous insert DNA and genomic DNA, or two (2) pieces of genomic DNA, or two (2) pieces of heterologous DNA. A “junction” is a point where two (2) specific DNA fragments join. For example, a junction exists where insert DNA joins flanking DNA. A junction point also exists in a transformed organism where two (2) DNA fragments join together in a manner that is modified from that found in the native organism. “Junction DNA” refers to DNA that comprises a junction point. Two junction sequences set forth in this disclosure are the junction point between the maize genomic DNA and the 5' end of the insert as set forth in SEQ ID NO: 27, and the junction point between the 3' end of the insert and maize genomic DNA as set forth in SEQ ID NO: 28.

As used herein, “heterologous” in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous nucleotide sequence can be from a species different from that from which the nucleotide sequence was derived, or, if from the same species, the promoter is not naturally found operably linked to the nucleotide sequence. A heterologous protein may originate from a foreign species, or, if from the same species, is substantially modified from its original form by deliberate human intervention.

“Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

“Promoter” refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements are often referred to as enhancers. Accordingly, an “enhancer” is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a

US 8,575,434 B2

9

native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters that cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okumuro and Goldberg (1989) *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, nucleic acid fragments of different lengths may have identical promoter activity.

The “translation leader sequence” refers to a nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect numerous parameters including, processing of the primary transcript to mRNA, mRNA stability and/or translation efficiency. Examples of translation leader sequences have been described (Turner and Foster (1995) *Mol. Biotechnol.* 3:225-236).

The “3' non-coding sequences” refer to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al. (1989) *Plant Cell* 1:671-680.

A “protein” or “polypeptide” is a chain of amino acids arranged in a specific order determined by the coding sequence in a polynucleotide encoding the polypeptide.

A DNA construct is an assembly of DNA molecules linked together that provide one or more expression cassettes. The DNA construct may be a plasmid that is enabled for self replication in a bacterial cell and contains various endonuclease enzyme restriction sites that are useful for introducing DNA molecules that provide functional genetic elements, i.e., promoters, introns, leaders, coding sequences, 3' termination regions, among others; or a DNA construct may be a linear assembly of DNA molecules, such as an expression cassette. The expression cassette contained within a DNA construct comprises the necessary genetic elements to provide transcription of a messenger RNA. The expression cassette can be designed to express in prokaryote cells or eukaryotic cells. Expression cassettes of the embodiments of the present invention are designed to express in plant cells.

The DNA molecules of embodiments of the invention are provided in expression cassettes for expression in an organism of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a coding sequence. “Operably linked” means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. Operably linked is intended to indicate a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. The cassette may additionally contain at least one additional gene to be co-transformed into the organism. Alternatively, the

10

additional gene(s) can be provided on multiple expression cassettes or multiple DNA constructs.

The expression cassette will include in the 5' to 3' direction of transcription: a transcriptional and translational initiation region, a coding region, and a transcriptional and translational termination region functional in the organism serving as a host. The transcriptional initiation region (i.e., the promoter) may be native or analogous, or foreign or heterologous to the host organism. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation.

It is to be understood that as used herein the term “transgenic” includes any cell, cell line, callus, tissue, plant part, or plant, the genotype of which has been altered by the presence of a heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term “transgenic” as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

A transgenic “event” is produced by transformation of plant cells with a heterologous DNA construct(s), including a nucleic acid expression cassette that comprises a transgene of interest, the regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. An event is characterized phenotypically by the expression of the transgene. At the genetic level, an event is part of the genetic makeup of a plant. The term “event” also refers to progeny produced by a sexual outcross between the transformant and another variety that include the heterologous DNA. Even after repeated back-crossing to a recurrent parent, the inserted DNA and flanking DNA from the transformed parent is present in the progeny of the cross at the same chromosomal location. The term “event” also refers to DNA from the original transformant comprising the inserted DNA and flanking sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

An insect resistant DP-004114-3 corn plant can be bred by first sexually crossing a first parental corn plant consisting of a corn plant grown from the transgenic DP-004114-3 corn plant and progeny thereof derived from transformation with the expression cassettes of the embodiments of the present invention that confers insect resistance, and a second parental corn plant that lacks insect resistance, thereby producing a plurality of first progeny plants; and then selecting a first progeny plant that is resistant to insects; and selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants an insect resistant plant. These steps can further include the back-crossing of the first insect resistant progeny plant or the second insect resistant progeny plant to the second parental corn plant or a third parental corn plant, thereby producing a corn plant that is resistant to insects.

As used herein, the term “plant” includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.),

US 8,575,434 B2

11

seeds, plant cells, and progeny of same. Parts of transgenic plants understood to be within the scope of the invention comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, leaves, and roots originating in transgenic plants or their progeny previously transformed with a DNA molecule of the invention and therefore consisting at least in part of transgenic cells, are also an embodiment of the present invention.

As used herein, the term "plant cell" includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants that can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

"Transformation" refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as "transgenic" organisms. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol.* 143:277) and particle-accelerated or "gene gun" transformation technology (Klein et al. (1987) *Nature* (London) 327:70-73; U.S. Pat. No. 4,945,050, incorporated herein by reference). Additional transformation methods are disclosed below.

Thus, isolated polynucleotides of the invention can be incorporated into recombinant constructs, typically DNA constructs, which are capable of introduction into and replication in a host cell. Such a construct can be a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., (1985; Supp. 1987) *Cloning Vectors: A Laboratory Manual*, Weissbach and Weissbach (1989) *Methods for Plant Molecular Biology*, (Academic Press, New York); and Flevin et al., (1990) *Plant Molecular Biology Manual*, (Kluwer Academic Publishers). Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several references, e.g., Fehr, in *Breeding Methods for Cultivar Development*, Wilcos J. ed., American Society of Agronomy, Madison Wis. (1987).

A "probe" is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. Such a probe is complementary to a strand of a target nucleic acid, in the case of the present invention, to a strand of

12

isolated DNA from corn event DP-004114-3 whether from a corn plant or from a sample that includes DNA from the event. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

"Primers" are isolated nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs of the invention refer to their use for amplification of a target nucleic acid sequence, e.g., by PCR or other conventional nucleic-acid amplification methods. "PCR" or "polymerase chain reaction" is a technique used for the amplification of specific DNA segments (see, U.S. Pat. Nos. 4,683,195 and 4,800,159; herein incorporated by reference).

Probes and primers are of sufficient nucleotide length to bind to the target DNA sequence specifically in the hybridization conditions or reaction conditions determined by the operator. This length may be of any length that is of sufficient length to be useful in a detection method of choice. Generally, 11 nucleotides or more in length, 18 nucleotides or more, and 22 nucleotides or more, are used. Such probes and primers hybridize specifically to a target sequence under high stringency hybridization conditions. Probes and primers according to embodiments of the present invention may have complete DNA sequence similarity of contiguous nucleotides with the target sequence, although probes differing from the target DNA sequence and that retain the ability to hybridize to target DNA sequences may be designed by conventional methods. Probes can be used as primers, but are generally designed to bind to the target DNA or RNA and are not used in an amplification process.

Specific primers can be used to amplify an integration fragment to produce an amplicon that can be used as a "specific probe" for identifying event DP-004114-3 in biological samples. When the probe is hybridized with the nucleic acids of a biological sample under conditions which allow for the binding of the probe to the sample, this binding can be detected and thus allow for an indication of the presence of event DP-004114-3 in the biological sample. Such identification of a bound probe has been described in the art. In an embodiment of the invention the specific probe is a sequence which, under optimized conditions, hybridizes specifically to a region within the 5' or 3' flanking region of the event and also comprises a part of the foreign DNA contiguous therewith. The specific probe may comprise a sequence of at least 80%, between 80 and 85%, between 85 and 90%, between 90 and 95%, and between 95 and 100% identical (or complementary) to a specific region of the event.

Methods for preparing and using probes and primers are described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed., vol. 1-3, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989 (hereinafter, "Sambrook et al., 1989"); Ausubel et al. eds., *Current Protocols in Molecular Biology*, Publishing and Wiley-Interscience, New York, 1995 (with periodic updates) (hereinafter, "Ausubel et al., 1995"); and Innis et al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as the PCR primer analysis tool in Vector NTI version 6 (Informax Inc., Bethesda Md.); PrimerSelect (DNASTAR Inc., Madison, Wis.); and Primer (Version 0.5©, 1991, Whitehead Institute

US 8,575,434 B2

13

for Biomedical Research, Cambridge, Mass.). Additionally, the sequence can be visually scanned and primers manually identified using guidelines known to one of skill in the art.

A “kit” as used herein refers to a set of reagents for the purpose of performing the method embodiments of the invention, more particularly, the identification of event DP-004114-3 in biological samples. The kit of the invention can be used, and its components can be specifically adjusted, for purposes of quality control (e.g. purity of seed lots), detection of event DP-004114-3 in plant material, or material comprising or derived from plant material, such as but not limited to food or feed products. “Plant material” as used herein refers to material which is obtained or derived from a plant.

Primers and probes based on the flanking DNA and insert sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed sequences by conventional methods, e.g., by re-cloning and sequencing such sequences. The nucleic acid probes and primers of the present invention hybridize under stringent conditions to a target DNA sequence. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. Nucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure.

A nucleic acid molecule is said to be the “complement” of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit “complete complementarity” when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be “minimally complementary” if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional “low-stringency” conditions. Similarly, the molecules are said to be “complementary” if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional “high-stringency” conditions. Conventional stringency conditions are described by Sambrook et al., 1989, and by Haymes et al., In: *Nucleic Acid Hybridization, a Practical Approach*, IRL Press, Washington, D.C. (1985), departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

In hybridization reactions, specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. The thermal melting point ( $T_m$ ) is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. For DNA-DNA hybrids, the  $T_m$  can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284:  $T_m = 81.5^\circ \text{C.} + 16.6 (\log M) + 0.41 (\% \text{ GC}) - 0.61 (\% \text{ form}) - 500/L$ ; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs.  $T_m$  is reduced by about  $1^\circ \text{C.}$  for each 1% of mismatching; thus,  $T_m$ , hybridization, and/or wash

14

conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the  $T_m$  can be decreased  $10^\circ \text{C.}$  Generally, stringent conditions are selected to be about  $5^\circ \text{C.}$  lower than the  $T_m$  for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or  $4^\circ \text{C.}$  lower than the  $T_m$ ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or  $10^\circ \text{C.}$  lower than the  $T_m$ ; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or  $20^\circ \text{C.}$  lower than the  $T_m$ .

Using the equation, hybridization and wash compositions, and desired  $T_m$ , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a  $T_m$  of less than  $45^\circ \text{C.}$  (aqueous solution) or  $32^\circ \text{C.}$  (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, N.Y.); and Ausubel et al., eds. (1995) and Sambrook et al. (1989).

As used herein, a substantially homologous sequence is a nucleic acid molecule that will specifically hybridize to the complement of the nucleic acid molecule to which it is being compared under high stringency conditions. Appropriate stringency conditions which promote DNA hybridization, for example,  $6\times$  sodium chloride/sodium citrate (SSC) at about  $45^\circ \text{C.}$ , followed by a wash of  $2\times \text{SSC}$  at  $50^\circ \text{C.}$ , are known to those skilled in the art or can be found in Ausubel et al. (1995), 6.3.1-6.3.6. Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about  $30^\circ \text{C.}$  for short probes (e.g., 10 to 50 nucleotides) and at least about  $60^\circ \text{C.}$  for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of a destabilizing agent such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at  $37^\circ \text{C.}$ , and a wash in  $1\times$  to  $2\times \text{SSC}$  ( $20\times \text{SSC} = 3.0 \text{ M NaCl}/0.3 \text{ M trisodium citrate}$ ) at  $50$  to  $55^\circ \text{C.}$  Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at  $37^\circ \text{C.}$ , and a wash in  $0.5\times$  to  $1\times \text{SSC}$  at  $55$  to  $60^\circ \text{C.}$  Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at  $37^\circ \text{C.}$ , and a wash in  $0.1\times \text{SSC}$  at  $60$  to  $65^\circ \text{C.}$  A nucleic acid of the invention may specifically hybridize to one or more of the nucleic acid molecules unique to the DP-004114-3 event or complements thereof or fragments of either under moderately stringent conditions.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local homology algorithm of Smith et al. (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.



US 8,575,434 B2

15

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0); the ALIGN PLUS program (version 3.0, copyright 1997); and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 10 (available from Accelrys, 9685 Scranton Road, San Diego, Calif. 92121, USA). Alignments using these programs can be performed using the default parameters.

The CLUSTAL program is well described by Higgins and Sharp, *Gene* 73: 237-244 (1988); Higgins and Sharp, *CABIOS* 5: 151-153 (1989); Corpet, et al., *Nucleic Acids Research* 16: 10881-90 (1988); Huang, et al., *Computer Applications in the Biosciences* 8: 155-65 (1992), and Pearson, et al., *Methods in Molecular Biology* 24: 307-331 (1994). The ALIGN and the ALIGN PLUS programs are based on the algorithm of Myers and Miller (1988) supra. The BLAST programs of Altschul et al. (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) supra. The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, Ausubel, et al., (1995). Alignment may also be performed manually by visual inspection.

To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al. (1997) supra. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used.

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity." Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

16

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

Regarding the amplification of a target nucleic acid sequence (e.g., by PCR) using a particular amplification primer pair, "stringent conditions" are conditions that permit the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product, the amplicon, in a DNA thermal amplification reaction.

The term "specific for (a target sequence)" indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

As used herein, "amplified DNA" or "amplicon" refers to the product of nucleic acid amplification of a target nucleic acid sequence that is part of a nucleic acid template. For example, to determine whether a corn plant resulting from a sexual cross contains transgenic event genomic DNA from the corn plant of the invention, DNA extracted from the corn plant tissue sample may be subjected to a nucleic acid amplification method using a DNA primer pair that includes a first primer derived from flanking sequence adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA. Alternatively, the second primer may be derived from the flanking sequence. The amplicon is of a length and has a sequence that is also diagnostic for the event. The amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. Alternatively, primer pairs can be derived from flanking sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence of the PHP27118 expression construct as well as the sequence flanking the transgenic insert. A member of a primer pair derived from the flanking sequence may be located a distance from the inserted DNA sequence, this distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about 20,000 bp. The use of the term "amplicon" specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

Nucleic acid amplification can be accomplished by any of the various nucleic acid amplification methods known in the art, including PCR. A variety of amplification methods are known in the art and are described, inter alia, in U.S. Pat. Nos. 4,683,195 and 4,683,202 and in Innis et al., (1990) supra. PCR amplification methods have been developed to amplify up to 22 Kb of genomic DNA and up to 42 Kb of bacteriophage DNA (Cheng et al., *Proc. Natl. Acad. Sci. USA* 91:5695-5699, 1994). These methods as well as other methods known in the art of DNA amplification may be used in the practice of the embodiments of the present invention. It is understood that a number of parameters in a specific PCR protocol may

US 8,575,434 B2

17

need to be adjusted to specific laboratory conditions and may be slightly modified and yet allow for the collection of similar results. These adjustments will be apparent to a person skilled in the art.

The amplicon produced by these methods may be detected by a plurality of techniques, including, but not limited to, Genetic Bit Analysis (Nikiforov, et al. *Nucleic Acid Res.* 22:4167-4175, 1994) where a DNA oligonucleotide is designed which overlaps both the adjacent flanking DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking sequence) a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Another detection method is the pyrosequencing technique as described by Winge (2000) *Innov. Pharma. Tech.* 00:18-24. In this method an oligonucleotide is designed that overlaps the adjacent DNA and insert DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. dNTPs are added individually and the incorporation results in a light signal which is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Fluorescence polarization as described by Chen et al., (1999) *Genome Res.* 9:492-498 is also a method that can be used to detect an amplicon of the invention. Using this method an oligonucleotide is designed which overlaps the flanking and inserted DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking DNA sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Taqman® (PE Applied Biosystems, Foster City, Calif.) is described as a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by the manufacturer. Briefly, a FRET oligonucleotide probe is designed which overlaps the flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

Molecular beacons have been described for use in sequence detection as described in Tyangi et al. (1996) *Nature Biotech.* 14:303-308. Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and

18

quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

A hybridization reaction using a probe specific to a sequence found within the amplicon is yet another method used to detect the amplicon produced by a PCR reaction.

Maize event DP-004114-3 is effective against insect pests including insects selected from the orders Coleoptera, Diptera, Hymenoptera, Lepidoptera, Mallophaga, Homoptera, Hemiptera, Orthoptera, Thysanoptera, Dermaptera, Isoptera, Anoplura, Siphonaptera, Trichoptera, etc., particularly Coleoptera and Lepidoptera.

Insects of the order Lepidoptera include, but are not limited to, armyworms, cutworms, loopers, and heliothines in the family Noctuidae: *Agrotis ipsilon* Hufnagel (black cutworm); *A. orthogonia* Morrison (western cutworm); *A. segetum* Denis & Schiffemüller (turnip moth); *A. subterranea* Fabricius (granulate cutworm); *Alabama argillacea* Hübner (cotton leaf worm); *Anticarsia gemmatilis* Hübner (velvetbean caterpillar); *Aethis mindara* Barnes and McDunnough (rough skinned cutworm); *Earias insulana* Boisduval (spiny bollworm); *E. vittella* Fabricius (spotted bollworm); *Egira* (*Xylomyges*) *curialis* Grote (citrus cutworm); *Euxoa messoria* Harris (darksided cutworm); *Helicoverpa armigera* Hübner (American bollworm); *H. zea* Boddie (corn earworm or cotton bollworm); *Heliothis virescens* Fabricius (tobacco budworm); *Hypena scabra* Fabricius (green cloverworm); *Hyponeuma taltula* Schaus; (*Mamestra configurata* Walker (bertha armyworm); *M. brassicae* Linnaeus (cabbage moth); *Melanchra picta* Harris (zebra caterpillar); *Mocis latipes* Guenée (small mocis moth); *Pseudaletia unipuncta* Haworth (armyworm); *Pseudoplusia includens* Walker (soybean looper); *Richia albicosta* Smith (Western bean cutworm); *Spodoptera frugiperda* J E Smith (fall armyworm); *S. exigua* Hübner (beet armyworm); *S. litura* Fabricius (tobacco cutworm, cluster caterpillar); *Trichoplusia ni* Hübner (cabbage looper); borers, casebearers, webworms, coneworms, and skeletonizers from the families Pyralidae and Crambidae such as *Achroia grisella* Fabricius (lesser wax moth); *Amyelois transitella* Walker (naval orangeworm); *Anagasta kuehniella* Zeller (Mediterranean flour moth); *Cadra cautella* Walker (almond moth); *Chilo partellus* Swinhoe (spotted stalk borer); *C. suppressalis* Walker (striped stem/rice borer); *C. terrellus* Pagenstecher (sugarcane stem borer); *Coryra cephalonica* Stainton (rice moth); *Crambus caliginosellus* Clemens (corn root webworm); *C. teterrellus* Zincken (bluegrass webworm); *Cnaphalocrocis medinalis* Guenée (rice leaf roller); *Desmia funeralis* Hübner (grape leaf folder); *Diaphania hyalinata* Linnaeus (melon worm); *D. nitidalis* Stoll (pickleworm); *Diatraea flavipennella* Box; *D. grandiosella* Dyar (southwestern corn borer); *D. saccharalis* Fabricius (sugarcane borer); *Elasmopalpus lignosellus* Zeller (lesser cornstalk borer); *Eoreuma loftini* Dyar (Mexican rice borer); *Ephestia elutella* Hübner (tobacco (cacao) moth); *Galleria mellonella* Linnaeus (greater wax moth); *Hedylepta accepta* Butler (sugarcane leafroller); *Herpetogramma licarsisalis* Walker (sod webworm); *Homoeosoma electellum* Hulst (sunflower moth); *Loxostege sticticalis* Linnaeus (beet webworm); *Maruca testulalis* Geyer (bean pod borer); *Orthaga thyrsalis* Walker (tea tree web moth); *Ostrinia nubi-*

US 8,575,434 B2

19

*lalis* Hübner (European corn borer); *Plodia interpunctella* Hübner (Indian meal moth); *Scirpophaga incertulas* Walker (yellow stem borer); *Udea rubigalis* Guenée (celery leaf-tier); and leaf rollers, budworms, seed worms, and fruit worms in the family Tortricidae *Acleris gloverana* Walsingham (Western blackheaded budworm); *A. variana* Fernald (Eastern blackheaded budworm); *Adoxophyes orana* Fischer von Rösslerstamm (summer fruit tortrix moth); *Archips* spp. including *A. argyrospila* Walker (fruit tree leaf roller) and *A. rosana* Linnaeus (European leaf roller); *Argyrotaenia* spp.; *Bonagota salubricola* Meyrick (Brazilian apple leafroller); *Choristoneura* spp.; *Cochylis hospes* Walsingham (banded sunflower moth); *Cydia latiferreana* Walsingham (filbert-worm); *C. pomonella* Linnaeus (codling moth); *Endopiza viteana* Clemens (grape berry moth); *Eupoecilia ambiguella* Hübner (vine moth); *Grapholita molesta* Busck (oriental fruit moth); *Lobesia botrana* Denis & Schifferrmüller (European grape vine moth); *Platynota flavedana* Clemens (variegated leafroller); *P. stultana* Walsingham (omnivorous leafroller); *Spilonota ocellana* Denis & Schifferrmüller (eyespotted bud moth); and *Suleima helianthana* Riley (sunflower bud moth).

Selected other agronomic pests in the order Lepidoptera include, but are not limited to, *Alsophila pometaria* Harris (fall cankerworm); *Anarsia lineatella* Zeller (peach twig borer); *Anisota senatoria* J. E. Smith (orange striped oak-worm); *Antheraea pernyi* Guérin-Ménéville (Chinese Oak Silkworm); *Bombyx mori* Linnaeus (Silkworm); *Bucculatrix thurberiella* Busck (cotton leaf perforator); *Collas eurytheme* Boisduval (alfalfa caterpillar); *Datana integerrima* Grote & Robinson (walnut caterpillar); *Dendrolimus sibiricus* Tschetwerikov (Siberian silk moth); *Ennomos subsignaria* Hübner (elm spanworm); *Erannis tiliaria* Harris (linden looper); *Erechthias flavistriata* Walsingham (sugarcane bud moth); *Euproctis chrysorrhoea* Linnaeus (brown tail moth); *Harrisina americana* Guérin-Ménéville (grapeleaf skeletonizer); *Heliothis subflexa* Guenée; *Hemileuca oliviae* Cockrell (range caterpillar); *Hypanthria cunea* Drury (fall webworm); *Keiferia lycopersicella* Walsingham (tomato pinworm); *Lambdina fiscellaria fiscellaria* Hulst (Eastern hemlock looper); *L. fiscellaria lugubrosa* Hulst (Western hemlock looper); *Leucoma salicis* Linnaeus (satin moth); *Lymantria dispar* Linnaeus (gypsy moth); *Malacosoma* spp.; *Manduca quinquemaculata* Haworth (five spotted hawk moth, tomato hornworm); *M. sexta* Haworth (tomato hornworm, tobacco hornworm); *Operophtera brumata* Linnaeus (winter moth); *Orgyia* spp.; *Paleacrita vernata* Peck (spring cankerworm); *Papilio cresphontes* Cramer (giant swallowtail, orange dog); *Phryganidia californica* Packard (California oakworm); *Phyllocnistis citrella* Stainton (citrus leafminer); *Phyllonorycter blancardella* Fabricius (spotted tentiform leafminer); *Pieris brassicae* Linnaeus (large white butterfly); *P. rapae* Linnaeus (small white butterfly); *P. napi* Linnaeus (green veined white butterfly); *Platyptilia carduidactyla* Riley (artichoke plume moth); *Plutella xylostella* Linnaeus (diamond-back moth); *Pectinophora gossypiella* Saunders (pink bollworm); *Pontia protodice* Boisduval & Leconte (Southern cabbageworm); *Sabulodes aegrotata* Guenée (omnivorous looper); *Schizura concinna* J. E. Smith (red humped caterpillar); *Sitotroga cerealella* Olivier (Angoumois grain moth); *Telchin licus* Drury (giant sugarcane borer); *Thaumetopoea pityocampa* Schifferrmüller (pine processionary caterpillar); *Tineola bisselliella* Hummel (webbing clothesmoth); *Tuta absoluta* Meyrick (tomato leafminer) and *Yponomeuta padella* Linnaeus (ermine moth).

Of interest are larvae and adults of the order Coleoptera including weevils from the families Anthribidae, Bruchidae, and Curculionidae including, but not limited to: *Anthonomus*

20

*grandis* Boheman (boll weevil); *Cylindrocopturus adspersus* LeConte (sunflower stem weevil); *Diaprepes abbreviatus* Linnaeus (Diaprepes root weevil); *Hypera punctata* Fabricius (clover leaf weevil); *Lissorhoptrus oryzophilus* Kuschel (rice water weevil); *Metamasius hemipterus hemipterus* Linnaeus (West Indian cane weevil); *M. hemipterus sericeus* Olivier (silky cane weevil); *Sitophilus granarius* Linnaeus (granary weevil); *S. oryzae* Linnaeus (rice weevil); *Smicronyx fulvus* LeConte (red sunflower seed weevil); *S. sordidus* LeConte (gray sunflower seed weevil); *Sphenophorus maidis* Chittenden (maize billbug); *S. livis* Vaurie (sugarcane weevil); *Rhabdoscelus obscurus* Boisduval (New Guinea sugarcane weevil); flea beetles, cucumber beetles, rootworms, leaf beetles, potato beetles, and leafminers in the family Chrysomelidae including, but not limited to: *Chaetocnema ectypa* Horn (desert corn flea beetle); *C. pulicaria* Melsheimer (corn flea beetle); *Colaspis brunnea* Fabricius (grape colaspis); *Diabrotica barberi* Smith & Lawrence (northern corn rootworm); *D. undecimpunctata howardi* Barber (southern corn rootworm); *D. virgifera virgifera* LeConte (western corn rootworm); *Leptinotarsa decemlineata* Say (Colorado potato beetle); *Oulema melanopus* Linnaeus (cereal leaf beetle); *Phyllotreta cruciferae* Goeze (corn flea beetle); *Zygogramma exclamationis* Fabricius (sunflower beetle); beetles from the family Coccinellidae including, but not limited to: *Epilachna varivestis* Mulsant (Mexican bean beetle); chafers and other beetles from the family Scarabaeidae including, but not limited to: *Antitrogonus parvulus* Britton (Childers cane grub); *Cyclocephala borealis* Arrow (northern masked chafer, white grub); *C. immaculata* Olivier (southern masked chafer, white grub); *Dermolepida albohirtum* Waterhouse (Greyback cane beetle); *Euethola humilis* rugiceps LeConte (sugarcane beetle); *Lepidiota frenchi* Blackburn (French's cane grub); *Tomarus gibbosus* De Geer (carrot beetle); *T. subtropicus* Blatchley (sugarcane grub); *Phyllophaga crinita* Burmeister (white grub); *P. latifrons* LeConte (June beetle); *Popillia japonica* Newman (Japanese beetle); *Rhizotrogus majalis* Razoumowsky (European chafer); carpet beetles from the family Dermestidae; wireworms from the family Elateridae, *Eleodes* spp., *Melanotus* spp. including *M. communis* Gyllenhal (wireworm); *Conoderus* spp.; *Limoniuss* spp.; *Agriotes* spp.; *Ctenicera* spp.; *Aeolus* spp.; bark beetles from the family Scolytidae; beetles from the family Tenebrionidae; beetles from the family Cerambycidae such as, but not limited to, *Migdolus fryanus* Westwood (longhorn beetle); and beetles from the Buprestidae family including, but not limited to, *Aphanisticus cochinchinae seminulum* Obenberger (leaf-mining buprestid beetle).

Adults and immatures of the order Diptera are of interest, including leafminers *Agromyza parvicornis* Loew (corn blotch leafminer); midges including, but not limited to: *Contarinia sorghicola* Coquillett (sorghum midge); *Mayetiola destructor* Say (Hessian fly); *Neolasioptera murtfeldtiana* Felt, (sunflower seed midge); *Sitodiplosis mosellana* Géhin (wheat midge); fruit flies (Tephritidae), *Oscinella frit* Linnaeus (frit flies); maggots including, but not limited to: *Delia* spp. including *Delia platura* Meigen (seedcorn maggot); *D. coarctata* Fallen (wheat bulb fly); *Fannia canicularis* Linnaeus, *F. femoralis* Stein (lesser house flies); *Meromyza americana* Fitch (wheat stem maggot); *Musca domestica* Linnaeus (house flies); *Stomoxys calcitrans* Linnaeus (stable flies); face flies, horn flies, blow flies, *Chrysomya* spp.; *Phormia* spp.; and other muscoid fly pests, horse flies *Tabanus* spp.; bot flies *Gastrophilus* spp.; *Oestrus* spp.; cattle grubs *Hypoderma* spp.; deer flies *Chrysops* spp.; *Melophagus ovinus* Linnaeus (keds); and other *Brachycera*, mosquitoes



US 8,575,434 B2

## 21

*Aedes* spp.; *Anopheles* spp.; *Culex* spp.; black flies *Prosimulium* spp.; *Simulium* spp.; biting midges, sand flies, sciarids, and other *Nematocera*.

Included as insects of interest are those of the order Hemiptera such as, but not limited to, the following families: Adelgidae, Aleyrodidae, Aphididae, Asterolecaniidae, Cercopidae, Cicadellidae, Cicadidae, Cixiidae, Coccidae, Coreidae, Dactylopiidae, Delphacidae, Diaspididae, Eriococcidae, Flatidae, Fulgoridae, Issidae, Lygaeidae, Margarodidae, Membracidae, Miridae, Ortheziidae, Pentatomidae, Phoenicococcidae, Phylloxeridae, Pseudococcidae, Psyllidae, Pyrrhocoridae and Tingidae.

Agronomically important members from the order Hemiptera include, but are not limited to: *Acrosternum hilare* Say (green stink bug); *Acyrtosiphon pisum* Harris (pea aphid); *Adelges* spp. (adelgids); *Adelphocoris rapidus* Say (rapid plant bug); *Anasa tristis* De Geer (squash bug); *Aphis craccivora* Koch (cowpea aphid); *A. fabae* Scopoli (black bean aphid); *A. gossypii* Glover (cotton aphid, melon aphid); *A. maidiradicis* Forbes (corn root aphid); *A. pomi* De Geer (apple aphid); *A. spiraeicola* Patch (spirea aphid); *Aulacaspis tegalensis* Zehntner (sugarcane scale); *Aulacorthum solani* Kaltenbach (foxglove aphid); *Bemisia tabaci* Gennadius (tobacco whitefly, sweetpotato whitefly); *B. argentifolii* Bellows & Perring (silverleaf whitefly); *Blissus leucopterus leucopterus* Say (chinch bug); *Blostomatidae* spp.; *Brevicoryne brassicae* Linnaeus (cabbage aphid); *Cacopsylla pyricola* Foerster (pear psylla); *Calocoris norvegicus* Gmelin (potato capsid bug); *Chaetosiphon fragaefolii* Cockerell (strawberry aphid); *Cimicidae* spp.; *Coreidae* spp.; *Corythuca gossypii* Fabricius (cotton lace bug); *Cyrtopeltis modesta* Distant (tomato bug); *C. notatus* Distant (suckfly); *Deois flavopicta* Stål (spittlebug); *Dialeurodes citri* Ashmead (citrus whitefly); *Diaphnocoris chlorionis* Say (honeysuckle plant bug); *Diuraphis noxia* Kurdjumov/Mordvilko (Russian wheat aphid); *Duplachionaspis divergens* Green (armored scale); *Dysaphis plantaginea* Paaserini (rosy apple aphid); *Dysdercus suturalis* Herrich-Schäffer (cotton stainer); *Dysmicoccus boninsis* Kuwana (gray sugarcane mealybug); *Empoasca fabae* Harris (potato leafhopper); *Eriosoma lanigerum* Hausmann (woolly apple aphid); *Erythroneura* spp. (grape leafhoppers); *Eumetopina flavipes* Muir (Island sugarcane planthopper); *Eurygaster* spp.; *Euschistus servus* Say (brown stink bug); *E. variolarius* Palisot de Beauvois (one-spotted stink bug); *Graptostethus* spp. (complex of seed bugs); and *Hyalopterus pruni* Geoffroy (mealy plum aphid); *Icerya purchasi* Maskell (cottony cushion scale); *Labopidicola allii* Knight (onion plant bug); *Laodelphax striatellus* Fallen (smaller brown planthopper); *Leptoglossus corculis* Say (leaf-footed pine seed bug); *Leptodictya tabida* Herrich-Schaeffer (sugarcane lace bug); *Lipaphis erysimi* Kaltenbach (turnip aphid); *Lygocoris pabulinus* Linnaeus (common green capsid); *Lygus lineolaris* Palisot de Beauvois (tarnished plant bug); *L. hesperus* Knight (Western tarnished plant bug); *L. pratensis* Linnaeus (common meadow bug); *L. rugulipennis* Poppius (European tarnished plant bug); *Macrosiphum euphorbiae* Thomas (potato aphid); *Macrosteles quadrilineatus* Forbes (aster leafhopper); *Magicauda septendecim* Linnaeus (periodical cicada); *Mahanarva fimbriolata* Stål (sugarcane spittlebug); *M. posticata* Stål (little cicada of sugarcane); *Melanaphis sacchari* Zehntner (sugarcane aphid); *Melanaspis glomerata* Green (black scale); *Metopolophium dirhodum* Walker (rose grain aphid); *Myzus persicae* Sulzer (peach-potato aphid, green peach aphid); *Nasonovia ribisnigri* Mosley (lettuce aphid); *Nephotettix cincticeps* Uhler (green leafhopper); *N. nigropictus* Stål (rice leafhopper); *Nezara viridula* Linnaeus (southern green stink bug); *Nilaparvata lugens* Stål (brown

## 22

planthopper); *Nysius ericae* Schilling (false chinch bug); *Nysius raphanus* Howard (false chinch bug); *Oebalus pugnax* Fabricius (rice stink bug); *Oncopeltus fasciatus* Dallas (large milkweed bug); *Orthops campestris* Linnaeus; Pemphigus spp. (root aphids and gall aphids); *Peregrinus maidis* Ashmead (corn planthopper); *Perkinsiella saccharicida* Kirkaldy (sugarcane delphacid); *Phylloxera devastatrix* Pergande (pecan phylloxera); *Planococcus citri* Risso (citrus mealybug); *Plesiocoris rugicollis* Fallen (apple capsid); *Poecilocapsus lineatus* Fabricius (four-lined plant bug); *Pseudatomoscelis seriatus* Reuter (cotton fleahopper); *Pseudococcus* spp. (other mealybug complex); *Pulvinaria elongata* Newstead (cottony grass scale); *Pyrilla perpusilla* Walker (sugarcane leafhopper); *Pyrrhocoridae* spp.; *Quadraspidiotus perniciosus* Comstock (San Jose scale); *Reduviidae* spp.; *Rhopalosiphum maidis* Fitch (corn leaf aphid); *R. padi* Linnaeus (bird cherry-oat aphid); *Saccharicoccus sacchari* Cockerell (pink sugarcane mealybug); *Scaptocoris castanea* Perty (brown root stink bug); *Schizaphis graminum* Rondani (greenbug); *Sipha flava* Forbes (yellow sugarcane aphid); *Sitobion avenae* Fabricius (English grain aphid); *Sogatella furcifera* Horvath (white-backed planthopper); *Sogatodes oryzicola* Muir (rice delphacid); *Spanagonicus albofasciatus* Reuter (whitemarked fleahopper); *Therioaphis maculata* Buckton (spotted alfalfa aphid); *Tinidae* spp.; *Toxoptera aurantii* Boyer de Fonscolombe (black citrus aphid); and *T. citricida* Kirkaldy (brown citrus aphid); *Trialeurodes abutiloneus* (bandedwinged whitefly) and *T. vaporariorum* Westwood (greenhouse whitefly); *Trioza diospyri* Ashmead (persimmon psylla); and *Typhlocyba pomaria* McAtee (white apple leafhopper).

Also included are adults and larvae of the order Acari (mites) such as *Aceria tosichella* Keifer (wheat curl mite); *Panonychus ulmi* Koch (European red mite); *Petrobia latens* Willer (brown wheat mite); *Steneotarsonemus bancrofti* Michael (sugarcane stalk mite); spider mites and red mites in the family Tetranychidae, *Oligonychus grypus* Baker & Pritchard, *O. indicus* Hirst (sugarcane leaf mite), *O. pratensis* Banks (Banks grass mite), *O. stickneyi* McGregor (sugarcane spider mite); *Tetranychus urticae* Koch (two spotted spider mite); *T. mcdanieli* McGregor (McDaniel mite); *T. cinnabarinus* Boisduval (carmine spider mite); *T. turkestanii* Ugarov & Nikolski (strawberry spider mite), flat mites in the family Tenuipalpidae, *Brevipalpus lewisi* McGregor (citrus flat mite); rust and bud mites in the family Eriophyidae and other foliar feeding mites and mites important in human and animal health, i.e. dust mites in the family Epidermoptidae, follicle mites in the family Demodicidae, grain mites in the family Glycyphagidae, ticks in the order Ixodidae, *Ixodes scapularis* Say (deer tick); *I. holocyclus* Neumann (Australian paralysis tick); *Dermacentor variabilis* Say (American dog tick); *Amblyomma americanum* Linnaeus (lone star tick); and scab and itch mites in the families Psoroptidae, Pyemotidae, and Sarcoptidae.

Insect pests of the order Thysanura are of interest, such as *Lepisma saccharina* Linnaeus (silverfish); *Thermobia domestica* Packard (firebrat).

Additional arthropod pests covered include: spiders in the order Araneae such as *Loxosceles reclusa* Gertsch & Mulaik (brown recluse spider); and the *Latrodectus mactans* Fabricius (black widow spider); and centipedes in the order Scutigermorpha such as *Scutigera coleoptrata* Linnaeus (house centipede). In addition, insect pests of the order Isoptera are of interest, including those of the termitidae family, such as, but not limited to, *Cornitermes cumulans* Kollar, *Cylindrotermes nordenskiöldi* Holmgren and *Pseudacanthotermes militaris* Hagen (sugarcane termite); as well as those in the



US 8,575,434 B2

23

Rhinotermitidae family including, but not limited to *Heterotermes tenuis* Hagen. Insects of the order Thysanoptera are also of interest, including but not limited to thrips, such as *Stenchaetothrips minutus* van Deventer (sugarcane thrips).

Embodiments of the present invention are further defined in the following Examples. It should be understood that these Examples are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the embodiments of the invention to adapt it to various usages and conditions. Thus, various modifications of the embodiments of the invention, in addition to those shown and described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

### EXAMPLES

#### Example 1

#### Transformation of Maize by *Agrobacterium* Transformation and Regeneration of Transgenic Plants Containing the Cry1F, Cry34Ab1, Cry35Ab1 (Cry34/35Ab1) and Pat Genes

4114 maize was produced by *Agrobacterium*-mediated transformation with plasmid PHP27118. This event contains the cry1F, cry34Ab1, cry35Ab1, and pat gene cassettes, which confer resistance to certain lepidopteran and coleopteran pests.

Specifically, the first cassette contains a truncated version of the cry1F gene from Bt var. *aizawai*. The insertion of the cry1F gene confers resistance to damage by lepidopteran pests, including ECB and FAW. The Cry1F protein (SEQ ID NO: 1) is comprised of 605 amino acids and has a molecular weight of approximately 68 kDa. The expression of the cry1F gene is controlled by the maize polyubiquitin promoter (Christensen et al., 1992, supra), providing constitutive

24

expression of Cry1F protein in maize. This region also includes the 5' UTR and intron associated with the native polyubiquitin promoter. The terminator for the cry1F gene is the poly(A) addition signal from open reading frame 25 (ORF 25) of the *Agrobacterium tumefaciens* (*A. tumefaciens*) Ti plasmid pTi15955 (Barker et al., 1983, supra).

The second cassette contains the cry34Ab1 gene isolated from Bt strain PS149B1 (U.S. Pat. Nos. 6,127,180; 6,624,145 and 6,340,593). The Cry34Ab1 protein (SEQ ID NO: 2) is 123 amino acid residues in length and has a molecular weight of approximately 14 kDa. The expression of the cry34Ab1 gene is controlled by a second copy of the maize polyubiquitin promoter with 5' UTR and intron (Christensen et al., 1992, supra). The terminator for the cry34Ab1 gene is the pinII terminator (Keil et al., 1986, supra; An et al., 1989, supra).

The third gene cassette contains the cry35Ab1 gene, also isolated from Bt strain PS149B1 (U.S. Pat. Nos. 6,083,499; 6,548,291 and 6,340,593). The Cry35Ab1 protein (SEQ ID NO: 3) has a length of 383 amino acids and a molecular weight of approximately 44 kDa. Simultaneous expression of the Cry34Ab1 and Cry35Ab1 proteins in the plant confers resistance to coleopteran insects, including WCRW. The expression of the cry35Ab1 gene is controlled by the *Triticum aestivum* (wheat) peroxidase promoter and leader sequence (Hertig et al. 1991, supra). The terminator for the cry35Ab1 gene is a second copy of the pinII terminator (Keil et al. 1986, supra; An et al. 1989, supra).

The fourth and final gene cassette contains a version of pat from *Streptomyces viridochromogenes* that has been optimized for expression in maize. The pat gene expresses PAT, which confers tolerance to phosphinothricin (glufosinate-ammonium). The PAT protein (SEQ ID NO: 4) is 183 amino acids residues in length and has a molecular weight of approximately 21 kDa. Expression of the pat gene is controlled by the promoter and terminator regions from the CaMV 35S transcript (Franck et al., 1980, supra; Odell et al., 1985, supra; Pietrzak, et al., 1986, supra). Plants containing the DNA constructs are also provided. A description of the genetic elements in the PHP27118 T-DNA (set forth in SEQ ID NO: 5) and their sources are described further in Table 1.

TABLE 1

Genetic Elements in the T-DNA Region of Plasmid PHP27118				
Location on T-DNA (bp position)	Genetic Element	Size (bp)	Description	
1 to 25	Right Border	25	T-DNA RB region from Ti plasmid of <i>A. tumefaciens</i>	
26 to 76	Ti Plasmid Region	51	Non-functional sequence from Ti plasmid of <i>A. tumefaciens</i>	
77 to 114	Polylinker Region	38	Region required for cloning genetic elements	
115 to 1014	ubiZM1 Promoter	900	Promoter region from <i>Zea mays</i> polyubiquitin gene (Christensen et al., 1992, supra)	
1015 to 1097	ubiZM1 5' UTR	83	5' UTR from <i>Zea mays</i> polyubiquitin gene. Id.	
1098 to 2107	ubiZM1 Intron	1010	Intron region from <i>Zea mays</i> polyubiquitin gene. Id.	
2108 to 2129	Polylinker Region	22	Region required for cloning genetic elements	
2130 to 3947	cry1F Gene	1818	Truncated version of cry1F from Bt var. <i>aizawai</i>	
3948 to 3992	Polylinker Region	45	Region required for cloning genetic elements	
3993 to 4706	ORF 25 Terminator	714	Terminator sequence from <i>A. tumefaciens</i> pTi15955 ORF 25 (Barker et al., 1983, supra)	
4707 to 4765	Polylinker Region	59	Region required for cloning genetic elements	

## US 8,575,434 B2

25

26

TABLE 1-continued

Genetic Elements in the T-DNA Region of Plasmid PHP27118			
Location on T-DNA (bp position)	Genetic Element	Size (bp)	Description
4766 to 5665	ubiZM1 Promoter	900	Promoter region from <i>Zea mays</i> polyubiquitin gene (Christensen et al., 1992, supra)
5666 to 5748	ubiZM1 5' UTR	83	5' UTR from <i>Zea mays</i> polyubiquitin gene. Id.
5749 to 6758	ubiZM1 Intron	1010	Intron region from <i>Zea mays</i> polyubiquitin gene. Id.
6759 to 6786	Polylinker Region	28	Region required for cloning genetic elements
6787 to 7158	cry34Ab1 Gene	372	Synthetic version of cry34Ab1 encoding 14 kDa delta-endotoxin parasporal crystal protein from the nonmotile strain PS149B1 of Bt (Moellenbeck et al. (2001) Nature Biotech. 19: 668-672; Ellis et al. (2002) Appl. Env. Microbiol. 68(3): 1137-1145; Herman et al. (2002) Environ. Entomol. 31(2): 208-214.)
7159 to 7182	Polylinker Region	24	Region required for cloning genetic elements
7183 to 7492	pinII Terminator	310	Terminator region from <i>Solanum tuberosum</i> proteinase inhibitor II gene (Keil et al. 1986, supra; An et al. 1989, supra)
7493 to 7522	Polylinker Region	30	Region required for cloning genetic elements
7523 to 8820	TA Peroxidase Promoter	1298	Promoter from <i>Triticum aestivum</i> peroxidase including leader sequence (Hertig et al. 1991, supra)
8821 to 8836	Polylinker Region	16	Region required for cloning genetic elements
8837 to 9988	cry35Ab1	1152	Synthetic version of cry35Ab1 encoding a 44 kDa delta-endotoxin parasporal crystal protein from the nonmotile strain PS149B1 of Bt (Moellenbeck et al. 2001, supra; Ellis et al. 2002, supra; Herman et al. 2002, supra)
9989 to 10012	Polylinker Region	24	Region required for cloning genetic elements
10013 to 10322	pinII Terminator	310	Terminator region from <i>Solanum tuberosum</i> proteinase inhibitor II gene (Keil et al. 1986, supra; An et al. 1989, supra)
10323 to 10367	Polylinker Region	45	Region required for cloning genetic elements
10368 to 10897	CaMV 35S Promoter	530	35S promoter from CaMV (Franck et al., 1980, supra; Odell et al., 1985, supra; Pietrzak, et al., 1986, supra)
10898 to 10916	Polylinker Region	19	Region required for cloning genetic elements
10917 to 11468	pat Gene	552	Synthetic, plant-optimized phosphinothricin acetyltransferase coding sequence from <i>Streptomyces viridochromogenes</i> .
11469 to 11488	Polylinker Region	20	Region required for cloning genetic elements
11489 to 11680	CaMV35S Terminator	192	35S terminator from CaMV (Franck et al., 1980, supra; Pietrzak, et al., 1986, supra)
11681 to 11756	Polylinker Region	76	Region required for cloning genetic elements
11757 to 11953	Ti Plasmid Region	197	Non-functional sequence from Ti plasmid of <i>A. tumefaciens</i>
11954 to 11978	Left Border	25	T-DNA LB region from Ti plasmid of <i>A. tumefaciens</i>

Immature embryos of maize (*Zea mays* L.) were aseptically removed from the developing caryopsis nine to eleven days after pollination and inoculated with *A. tumefaciens* strain LBA4404 containing plasmid PHP27118 (FIG. 1), essentially as described in Zhao (U.S. Pat. No. 5,981,840, the contents of which are hereby incorporated by reference). The T-DNA region of PHP27118 is shown in FIG. 2. After three to six days of embryo and *Agrobacterium* co-cultivation on solid culture medium with no selection, the embryos were then transferred to a medium without herbicide selection but containing carbenicillin. After three to five days on this medium, embryos were then transferred to selective medium that was stimulatory to maize somatic embryogenesis and contained bialaphos for selection of cells expressing the pat transgene.

The medium also contained carbenicillin to kill any remaining *Agrobacterium*. After six to eight weeks on the selective medium, healthy, growing calli that demonstrated resistance to bialaphos were identified. The putative transgenic calli were subsequently regenerated to produce T0 plantlets.

Samples were taken from the T0 plantlets for PCR analysis to verify the presence and copy number of the inserted cry1F, cry35Ab1, cry34Ab1, and/or pat genes. Maize event DP-004114-3 was confirmed to contain a single copy of the T-DNA (See Examples 2 and 3). In addition to this analysis, the T0 plantlets were analyzed for the presence of certain *Agrobacterium* binary vector backbone sequences by PCR (data not shown). Plants that were determined to be single copy for the inserted genes and negative for *Agrobacterium*

US 8,575,434 B2

27

backbone sequences were selected for further greenhouse propagation. These selected T0 plants were screened for trait efficacy and protein expression by conducting numerous bioassays (See Example 5). The T0 plants meeting all criteria were advanced and crossed to inbred lines to produce seed for further testing. A schematic overview of the transformation and event development is presented in FIG. 3.

## Example 2

## Identification of Maize Event DP-004114-3

Genomic DNA from leaf tissue of test seed from 4114 maize and a control substance (seed from a non-genetically modified maize with a genetic background representative of the event background) was isolated and subjected to qualitative PCR amplification using a construct-specific primer pair. The PCR products were separated on an agarose gel to confirm the presence of the inserted construct in the genomic DNA isolated from the test seed, and the absence of the inserted construct in the genomic DNA isolated from the control seed. A reference standard (Low DNA Mass Ladder; Invitrogen Corporation Catalog #10380-012) was used to determine the PCR product size. The reliability of the construct-specific PCR method was assessed by repeating the experiment three times. The sensitivity of the PCR amplification was evaluated by various dilutions of the genomic DNA from 4114 maize.

Test and control leaf samples (V5-V7 leaf stage) were harvested from plants grown at the DuPont Experimental Station (Wilmington, Del.) from seed obtained from Pioneer Hi-Bred (Johnston, Iowa). Genomic DNA extractions from the test and control leaf tissues were performed using a standard urea extraction protocol.

Genomic DNA was quantified using the NanoDrop 1000 Spectrophotometer using ND-1000 V3.6 Software (Thermo-Scientific, Wilmington, Del.) and the Quant-iT PicoGreen® reagent (Invitrogen, Carlsbad, Calif.). DNA samples were visualized on an agarose gel to confirm quantitation values and to determine the DNA quality.

Genomic DNA samples isolated from leaf tissue of 4114 maize and control samples were subjected to PCR amplification (Roche High Fidelity PCR Master Kit, Roche Catalog #12140314001) utilizing a construct-specific primer pair (SEQ ID NOs: 7 and 8) which spans the maize ORF 25 terminator and the ubiquitin promoter (See FIG. 2), and allows for the unique identification of the inserted T-DNA in 4114 maize. A second primer set (SEQ ID NOs: 9 and 10) was used to amplify the endogenous maize invertase gene (GenBank accession number AF171874.1) as a positive control for PCR amplification. The PCR target site and size of the expected PCR product for each primer set are shown in Table 2. PCR reagents and reaction conditions are shown in Table 3. In this study, 50 ng of leaf genomic DNA was used in all PCR reactions.

TABLE 2

PCR Genomic DNA Target Site and Expected Size of PCR Products		
Primer Set	Target Site	Expected Size of PCR Product (bp)
SEQ ID NO: 7 & 8	Construct Specific T-DNA: ORF 25 terminator and ubiquitin promoter	287
SEQ ID NO: 9 & 10	Endogenous maize invertase gene	225

28

TABLE 3

PCR Reagents and Reaction Conditions					
PCR Reagents		PCR Reaction Conditions			
Reagent	Volume (μL)	Cycle Element	Temp (° C.)	Time (sec)	# Cycles
Template DNA (25 ng/μL)	2	Initial Denaturation	94	120	1
Primer 1 (10 μM)	2	Denaturation	94	10	35
Primer 2 (10 μM)	2	Annealing	65	15	
PCR Master Mix*	25	Elongation	68	60	
ddH <sub>2</sub> O	19	Final Elongation	68	420	1
—	—	Hold Cycle	4	Until analysis	—

ddH<sub>2</sub>O: double-distilled water

\*Roche High Fidelity Master Mix

A PCR product of approximately 300 bp in size amplified by the construct-specific primer set (SEQ ID NOs: 7 and 8) was observed in PCR reactions using plasmid PHP27118 (10 ng) as a template and all 4114 maize DNA samples, but absent in all control maize samples and the no-template control. This experiment was repeated three times, and similar results were obtained. Results observed for DNA extracts from five 4114 maize plants and five control maize plants corresponded closely with the expected PCR product size (287 bp) for samples containing 4114 maize genomic DNA. A PCR product approximately 220 bp in size was observed for both 4114 maize and control maize samples following PCR reaction with the primer set (SEQ ID NOs: 9 and 10) for detection of the endogenous maize invertase gene. These results corresponded closely with the expected PCR product size (225 bp) for genomic DNA samples containing the maize endogenous invertase gene. The endogenous target band was not observed in the no-template control.

In order to assess the sensitivity of the PCR amplification, various concentrations of a single DNA sample from 4114 maize were diluted in non-genetically modified control DNA, resulting in 4114 maize DNA amounts ranging from 500 fg, 5 pg, 10 pg, 50 pg, 100 pg, 500 pg, 5 ng, and 50 ng (the total amount of genomic DNA in all PCR samples was 50 ng). Each dilution was subjected to PCR amplification as previously conducted. Based on this analysis, the limit of detection (LOD) was determined to be approximately 100 pg of 4114 maize DNA in 50 ng of total DNA, or 0.2% 4114 maize DNA.

In conclusion, qualitative PCR analysis utilizing a construct-specific primer set for 4114 maize confirmed that the test plants contained the inserted T-DNA from plasmid PHP27118, as evident by the presence of the construct-specific target band in all test plant samples analyzed, and the absence in the non-genetically modified control plants. This result was reproducible. Test and control plants both contained the endogenous maize invertase gene. The sensitivity of the analysis under the conditions described is approximately 100 pg of 4114 maize genomic DNA in 50 ng of total genomic DNA or 0.2% 4114 maize genomic DNA.

## Example 3

## Southern Blot Analysis of DP-004114-3 Maize for Integrity and Copy Number

Southern blot analysis was used to confirm the integrity and copy number of the inserted T-DNA from PHP27118 and to confirm the presence of the cry1F, cry34Ab1, cry35Ab1, and pat gene cassettes in 4114 maize.

US 8,575,434 B2

29

Five individual plants from the T1 generation of 4114 maize were selected for Southern blot analysis. Young leaf material was harvested from the 4114 maize (test) and non-transgenic maize (control) plants and was immediately placed on dry ice. The frozen samples were lyophilized and genomic DNA was extracted from the test and control tissues using a CTAB extraction method.

Following restriction enzyme digestions as detailed below, the DNA fragments were separated on agarose gels, depurinated, denatured, and neutralized in situ, and transferred to a nylon membrane in 20xSSC buffer using the method as described for TURBOBLOTTER™ Rapid Downward Transfer System (Schleicher & Schuell). Following transfer to the membrane, the DNA was bound to the membrane by ultra-violet light crosslinking.

#### Integrity

The restriction enzyme Hind III was selected for Southern analysis of integrity, as there are three sites located within the T-DNA (FIG. 2). Approximately 1-3 µg of genomic DNA was digested with Hind III and separated by size on an agarose gel. As a positive control, approximately 15 pg of plasmid containing the PHP27118 T-DNA was spiked into a control plant DNA sample, digested and included on the agarose gel. A negative control was also included to verify background hybridization of the probe to the maize genome.

Four probes homologous to the cry1F, cry34Ab1, cry35Ab1, and pat genes on the PHP27118 T-DNA (for gene elements, see FIG. 2) were used for hybridization to confirm the presence of the genes. In order to develop the probes, fragments homologous to the cry1F, cry34Ab1, cry35Ab1, and pat genes were generated by PCR from plasmid containing the PHP27118 T-DNA, size separated on an agarose gel, and purified using a QIAquick® gel extraction kit (Qiagen). All DNA probes were subsequently generated from the fragments using the Rediprime™ II DNA Labeling System (Amersham) which performs random prime labeling with [<sup>32</sup>P] dCTP.

The labeled probes were hybridized to the target DNA on the nylon membranes for detection of the specific fragments using the MiracleHyb® Hybridization Solution essentially as described by the manufacturer (Stratagene). Washes after hybridization were carried out at high stringency. Blots were exposed to X-ray film at -80° C. for one or more time points to detect hybridizing fragments.

Because the Hind III enzyme sites were known within the T-DNA, exact expected band sizes were determined for each of the probes (Table 4, FIG. 2). For an intact copy of the T-DNA, the cry1F probe was expected to hybridize to a fragment of 3891 bp. The cry34Ab1, cry35Ab1, and pat gene probes were expected to hybridize to a fragment of 7769 bp. Fragments from the test samples matching the expected sizes, as well as matching the bands in the plasmid control sample, would confirm the integrity of the inserted T-DNA and the presence of each gene.

The results of the Southern blot analysis with Hind III and the cry1F, cry34Ab1, cry35Ab1, and pat gene probes confirmed the expected fragment sizes and, thus, confirmed that the T-DNA inserted intact into each of the events and that each of the genes was present.

A band of approximately 4 kb was observed with the cry1F probe which is consistent with the expected fragment size. A similar fragment of approximately 4 kb was observed in the plasmid positive control lane, which was presumed to be the expected band of 3891 bp. Based on equivalent migration of the hybridizing band in the events to the band in the plasmid positive control, it was confirmed that the portion of the T-DNA containing cry1F had inserted intact in 4114 maize.

30

In the hybridization with the cry34Ab1 probe, a band of approximately 8 kb was observed in the event and also in the plasmid positive control. The hybridizing band in the plasmid positive control lane was presumed to be the expected band of 7769 bp. Because the hybridizing band in the event had migrated equivalently with this band, it was confirmed that this portion of the T-DNA containing cry34Ab1 was inserted intact.

Similarly, hybridizations with cry35Ab1 and pat hybridized to the same 7769 bp fragment in the plant and plasmid positive control as expected. These results confirmed that the portion of the T-DNA containing the cry35Ab1 and pat genes had inserted intact.

This Southern blot analysis confirms that 4114 maize contains an intact copy of the T-DNA from PHP27118 containing the cry1F, cry34Ab1, cry35Ab1, and pat genes.

TABLE 4

Summary of Expected and Observed Hybridization Fragments on Southern Blots for 4114 Maize DNA digested with Hind III

Probe	Expected Fragment Size from PHP27118 T-DNA (bp) <sup>1</sup>	Observed Fragment Size (kb) <sup>2</sup>
cry1F	3891	~4
cry34Ab1	7769	~8
cry35Ab1	7769	~8
pat	7769	~8

<sup>1</sup>Expected fragment sizes based on map of PHP27118 T-DNA (FIG. 2).

<sup>2</sup>All observed fragments migrated equivalently with the plasmid positive control and, therefore, were confirmed to represent the intact portion of the PHP27118 T-DNA.

#### Copy Number

The cry1F and pat probes were used in Southern blot hybridizations to evaluate the copy number of the insertions in 4114 maize.

The restriction enzyme Bcl I was selected for Southern analysis of copy number, as there is a single site located within the T-DNA (FIG. 2). Approximately 3 µg of genomic DNA from individual plants of the T1 generation of event 4114 was digested with Bcl I and separated by size on an agarose gel. A plasmid containing the PHP27118 T-DNA was spiked into a control plant DNA sample, digested and included on the agarose gel to serve as a positive hybridization control. Negative control maize DNA was also included to verify background hybridization of the probe to the maize genome. DNA Molecular Weight Marker VII, digoxigenin (DIG) labeled (Roche, Indianapolis, Ind.), was included on Bcl I blots as a size standard for hybridizing fragments.

Probes for the cry1F and pat genes were also labeled by a PCR reaction incorporating a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, into the fragment. PCR labeling of isolated fragments was carried out according to the procedures supplied in the PCR DIG Probe Synthesis Kit (Roche).

The DIG-labeled probes were hybridized to the Bcl I Southern blots of the T1 generation of the 4114 event. Probes were hybridized to the target DNA for detection of the specific fragments using DIG Easy Hyb solution (Roche) essentially as described by manufacturer. Post-hybridization washes were carried out at high stringency. DIG-labeled probes hybridized to the bound fragments were detected using the CDP-Star Chemiluminescent Nucleic Acid Detection System (Roche). Blots were exposed to X-ray film at room temperature for one or more time points to detect



US 8,575,434 B2

31

hybridizing fragments. Membranes were stripped of hybridized probe following the manufacturer's recommendation prior to hybridization with additional probes.

The restriction enzyme Bcl I, having a single restriction site within the T-DNA (FIG. 2), was selected to confirm the presence of a single PHP27118 T-DNA insertion in 4114 maize. The site for Bcl I is located at by 2546 of the T-DNA (FIG. 2) and will yield fragments of greater than about 2500 bp and 9400 bp for a single inserted T-DNA. Hybridization with the pat probe would indicate the number of copies of this element found in the event based on the number of hybridizing bands (e.g., one hybridizing band indicates one copy of the element). The pat probe would hybridize to the fragment of greater than 9400 bp. Because the Bcl I restriction enzyme site is within the cry1F gene, the cry1F probe is expected to hybridize to both fragments and result in two bands for a single T-DNA insertion (FIG. 2).

The results of the Southern blot analysis with Bcl I and the cry1F and pat gene probes for 4114 maize are summarized in Table 5.

TABLE 5

Summary of Expected and Observed Hybridization Fragments on Southern Blots for Bcl I digests of 4114 Maize			
Probe	Enzyme Digest	Expected Fragment Size from PHP27118 T-DNA (bp) <sup>1</sup>	Observed Fragment Size (kb) <sup>2</sup>
cry1F	Bcl I	>2500 <sup>3</sup>	~3.1
		>9400	>8.6
pat	Bcl I	>9400	>8.6

<sup>1</sup>Expected fragment sizes based on map of PHP27118 T-DNA (FIG. 2).

<sup>2</sup>All observed fragment sizes are approximated based on the migration of the DIG VII molecular weight marker.

<sup>3</sup>Two fragments are expected with the cry1F probe due to the location of the Bcl I restriction site within the cry1F gene.

The results of the Southern blot analysis of 4114 maize with Bcl I digestion and the cry1F probe showed two bands as expected, one band of greater than 8.6 kb and a second band of approximately 3.1 kb. Two bands are expected for a single insertion due to the location of the Bcl I site within the cry1F gene, so these results indicate that there is a single copy of cry1F in 4114 maize. The results of the Southern blot analysis of 4114 maize with Bcl I digestion and the pat probe showed a single band of greater than 8.6 kb that matched the size of the larger cry1F band as expected. These results indicate that there is also a single insertion of the pat gene in maize event 4114.

As the cry34Ab1 and cry35Ab1 genes are located on the same fragment as the pat gene and part of the cry1F gene, and between the cry1F and pat genes on the T-DNA, by extension this also demonstrates that this event is likely to contain a single copy of each of these genes.

#### Example 4

##### Sequencing Characterization of Insert and Genomic Border Regions of Maize Event DP-004114-3

The sequence of the insert and genomic border regions was determined to confirm the integrity of the inserted DNA and to characterize the genomic sequence flanking the insertion site present in 4114 maize. In total, 16,752 bp of 4114 maize genomic sequence was confirmed, comprising 2,422 bp of the 5' genomic border sequence, 2,405 bp of the 3' genomic

32

border sequence, and 11,925 bp of inserted T-DNA from PHP27118. The inserted T-DNA in 4114 maize was found to have a 29 bp deletion on the Right Border (RB) end and a 24 bp deletion on the Left Border (LB) end. All remaining sequence is intact and identical to that of plasmid PHP27118. The 5' and 3' genomic border regions of 4114 maize were verified to be of maize origin by PCR amplification and sequencing of the genomic border regions from both 4114 maize and control maize plants.

Seed containing event DP-004114-3 was obtained from a T1S2 generation of 4114 maize. Control seed was obtained from a maize line that has a similar genetic background to 4114 maize but does not contain the cry1F, cry34Ab1, cry35Ab1, and pat gene cassettes. All seeds were obtained from Pioneer Hi-Bred International, Inc. (Johnston, Iowa). The Low DNA Mass Ladder (Invitrogen Corp., Carlsbad, Calif.) and the High DNA Mass Ladder (Invitrogen Corp.) were used for gel electrophoresis to estimate DNA fragment sizes on agarose gels.

The 4114 maize seed and the control seed were planted in growth chambers at the DuPont Experimental Station (Wilmington, Del.) to produce plant tissues used for this study. One seed was planted per pot, and the pot was uniquely identified. All plants were grown with light, temperature, and water regulated for healthy plant growth. Leaf samples were collected from the control and 4114 maize plants. For each individual plant, leaf material was collected in a pre-labeled bag, placed on dry ice, and then transferred to an ultra low freezer (<-55° C.) following collection. All samples were maintained frozen until tissue processing.

##### Genotype Confirmation Via Event-Specific PCR Analysis

A leaf sample was taken from all test and control plants for event-specific PCR analysis. DNA was extracted from each leaf sample using the Extract-N-Amp™ Plant PCR kit following the described procedure (Sigma-Aldrich, St. Louis, Mo.) for real-time PCR analysis.

Real-time PCR was performed on each DNA sample utilizing an ABI PRISM® 7500HT Sequence Detection System (Applied Biosystems, Inc., Foster City, Calif.). TaqMan® probe (Applied Biosystems, Inc.) and primer sets (Integrated DNA Technologies, Coralville, Iowa) were designed to detect a target sequence from 4114 maize. In addition, a second TaqMan® probe and primer set for a reference maize endogenous gene was used to confirm the presence of amplifiable DNA in each reaction. The analysis consisted of real-time PCR determination of qualitative positive/negative calls. The extracted DNA was assayed using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems, Inc.).

Positive or negative determination for 4114 maize was based on comparison of the CT (threshold cycle) of the event-specific target PCR to that of the maize endogenous reference target. If the event and endogenous PCR targets amplified above CT threshold, then the plant was scored as positive for that event. If the endogenous target amplified and the event target did not, then the plant was scored as negative. If neither target amplified for a particular sample, then it was determined to be a poor quality DNA sample or failed run and the assay was repeated.

All 4114 maize plants were positive for the event-specific PCR and the PAT, Cry1F, and Cry34Ab1 proteins, whereas all the control maize plants were negative. The results are summarized in Table 6.

US 8,575,434 B2

33

TABLE 6

Summary of Event-Specific PCR Analysis and Cry1F, Cry34Ab1, and PAT Protein Expression in 4114 Maize and Control Maize Plants				
4114 Maize Plant ID	Event-Specific PCR <sup>1</sup>	Cry1F <sup>2</sup>	Cry34Ab1 <sup>2</sup>	PAT <sup>2</sup>
T-F-08-233C-1	+	+	+	+
T-F-08-233C-2	+	+	+	+
T-F-08-233C-3	+	+	+	+
T-F-08-233C-4	+	+	+	+
Control Maize Plant ID				
C-F-08-246C-1	-	-	-	-
C-F-08-246C-2	-	-	-	-

<sup>1</sup>Summary of event-specific real time PCR assay for 4114 maize. Positive (+) indicates the presence of 4114 maize event. Negative (-) indicates the absence of 4114 maize event.

<sup>2</sup>Summary of Cry1F, Cry34Ab1, and PAT protein expression in 4114 maize and control maize plants using lateral flow devices. Positive (+) indicates the presence of the protein. Negative (-) indicates the absence of the protein.

#### DNA Sequencing

DNA fragments were cloned and submitted for sequencing at the Pioneer Crop Genetics Research sequencing facility (Wilmington, Del.). Sequencher™ software from Gene Codes Corporation (Ann Arbor, Mich.) was used to assemble the sequences. Sequence annotation was performed using Vector NTI 9.1.0 (Invitrogen Corp) by comparing the T-DNA insert sequences generated from 4114 maize with the sequences from the T-DNA region of plasmid PHP27118 (used for transformation to produce 4114 maize).

The T-DNA region of plasmid PHP27118, used to create 4114 maize, was sequenced and compared with the inserted T-DNA sequence in 4114 maize.

The sequence of the T-DNA region of plasmid PHP27118 was used to design primer pairs to characterize the inserted T-DNA in 4114 maize. Six overlapping PCR products were generated using genomic DNA from four different 4114 maize plants as template. These PCR products were cloned and sequenced.

#### Sequencing of 5' and 3' Flanking Genomic Border Regions

Preliminary sequence characterization of the 5' and 3' flanking genomic border regions were carried out using several rounds of inverse PCR, (Silver and Keerikatte (1989) *J.*

34

*Virol.* 63: 1924; Ochman et al. (1988) *Genetics* 120:621-623; Triglia et al., (1988) *Nucl. Acids Res.* 16:8186) with primers anchored within various regions of the inserted T-DNA. Sequence information obtained from inverse PCR was subjected to BLASTn analysis and showed a match to the maize BAC clone AC211214 from the NCBI (National Center for Biotechnology Information) GenBank nucleotide database. This sequence was then used to design primers that spanned the 5' and 3' insert/genomic junctions in 4114 maize. The PCR products generated from four 4114 maize plants were cloned and sequenced to verify the 5' and 3' insert/genomic junctions and the genomic border regions.

In addition, to demonstrate that the identified 5' and 3' genomic border regions were of maize origin, PCR was performed on 4114 maize and control maize plants within the genomic regions. Each PCR fragment was directly sequenced to verify its identity of maize origin.

The T-DNA sequence information of plasmid PHP27118 was used to design primers to verify the inserted sequence in 4114 maize (Tables 7 and 8).

TABLE 7

PCR Primers Used to Characterize the Genomic Border Regions and Inserted T-DNA in 4114 Maize				
PCR Fragment	Primer Pair	Primer SEQ ID NOs:	Size (bp)	Amplified Region
A	09-0-3030/ 09-0-2787	11/12	2511	5' Genomic border region and insert
B	09-0-3036/ 09-0-3046	13/14	3622	Insert
C	09-0-2980/ 09-0-3045	16/15	4146	Insert
D	08-0-2463/ 08-0-2759	17/18	2713	Insert
E	09-0-2775/ 09-0-3083	19/20	3062	Insert
F	09-0-2799/ 09-0-3005	21/22	2612	3' Genomic border region and insert
G	09-0-3230/ 09-0-3229	23/24	257	5' Genomic border region
H	09-0-3231/ 09-0-3084	25/26	283	3' Genomic border region

TABLE 8

Sequence and Location of Primers Used For PCR Reactions.			
PCR Fragment	Primer (SEQ ID NO:)	Primer Sequence (5'-3')	Target Sequence Location (bp to bp) <sup>1</sup>
A	09-0-3030 (SID: 11)	GAGCATATCCAGCACCAGCTGGTACCAAG	1-29
	09-0-2787 (SID: 12)	GCAGGCATGCCCCGCGATA	2,511-2,493
B	09-0-3036 (SID: 13)	TGGTCTACCCGATGATGTGATTGGCC	1,994-2,019
	09-0-3046 (SID: 14)	CGAAGACAGGATCTGACAAGGTCGATAG	5,615-5,587
C	09-0-3045 (SID: 15)	GACTTCATGAACCTTTGTTGTGACTGCAGAGAC	5,414-5,414
	09-0-2980 (SID: 16)	CTCATGACTCAGGACTTGTGGC	9,559-9,538
D	08-0-2463 (SID: 17)	ATCAGCCTCTACTTCGAC	9,390-9,407
	08-0-2759 (SID: 18)	CTCCATGATCTTCGTCTCATGTG	12,102-12,080

US 8,575,434 B2

35

TABLE 8-continued

Sequence and Location of Primers Used For PCR Reactions.			
PCR Fragment	Primer (SEQ ID NO:)	Primer Sequence (5'-3')	Target Sequence Location (bp to bp) <sup>1</sup>
E	09-0-2775	CACCAACTCCATCCAGAAGTGGC	11,481-
	(SID: 19)		11,503
	09-0-3083	GCCTTGCCATTGGCGCAGTGAGAACCG	14,542-
	(SID: 20)		14,517
F	09-0-2799	CGGCGCGCCTCTAGTTGAAGACACGTT	14,141-
	(SID: 21)		14,167
	09-0-3005	CAGTGGACTGAGCCGACAGCTAAGGACAC	16,752-
	(SID: 22)		16,723
G	09-0-3230	GGAACATTGAGCTTGGGAGTCTGGACT	2,086-2,113
	(SID: 23)		
	09-0-3229	GAACAGGGTCCTCGAATCAAGGGCAGC	2,342-2,316
	(SID: 24)		
H	09-0-3231	CGGTTCTCACTGCGCCAATGCAAGGC	14,517-
	(SID: 25)		14,542
	09-0-3084	CATGACGACCATGAAGCAACATC	14,799-
	(SID: 26)		14,777

<sup>1</sup>Location in sequence of 4114 Maize.

Bases 1-2,422 = 5' genomic border region

Bases 2,423-14,347 = insert

Bases 14,348-16,752 = 3' genomic border region.

To characterize the inserted T-DNA in 4114 maize, PCR primers were designed to amplify the T-DNA insert in six separate, overlapping PCR products as outlined in Table 7: fragments A through F (Positions indicated in FIG. 5). As expected, the predicted PCR products were generated only from 4114 maize genomic DNA samples, and were not present in the control maize samples. The six PCR products were cloned and sequenced. When comparing the sequence of the inserted T-DNA in 4114 maize to the T-DNA region of plasmid PHP27118 used to create 4114 maize, it was determined that there was a 29 bp deletion on the RB end, and a 24 bp deletion on the LB end. RB and LB termini deletions often occur in *Agrobacterium*-mediated transformation (Kim et al. (2007) *Plant J.* 51:779-791). All remaining sequence is intact and identical to that of plasmid PHP27118. The sequence of the insertion is presented in SEQ ID NO: 6.

To verify the additional 5' genomic border sequence, PCR was performed with a forward primer (SEQ ID NO: 11) in the 5' genomic border region and a reverse primer (SEQ ID NO: 12) within the inserted T-DNA. The resulting 2,511 bp PCR fragment A from 4114 maize genomic DNA samples was cloned and sequenced (FIG. 3). The 2,422 bp of the 5' genomic border region sequence is set forth in nucleotides 1-2,422 of SEQ ID NO: 6.

To verify the additional 3' genomic border sequence, PCR was performed with a forward primer (SEQ ID NO: 21) within the inserted T-DNA and a reverse primer (SEQ ID NO: 22) in the 3' genomic border region. The resulting 2,612 bp PCR fragment F from 4114 maize genomic DNA samples was cloned and sequenced (FIG. 3). The 2,405 bp of the 3' genomic border region sequence is set forth in nucleotides 14,348 to 16,752 of SEQ ID NO: 6.

In total, 16,752 bp of sequence from genomic DNA of 4114 maize were confirmed: 2,422 bp of the 5' genomic border sequence, 2,405 bp of the 3' genomic border sequence, and 11,925 bp comprising the inserted T-DNA.

To demonstrate that the identified 5' and 3' flanking genomic border sequences are of maize origin, PCR was

36

performed within the 5' and 3' genomic border regions (the primer pair set forth in SEQ ID NOs: 23 and 24 and the primer pair set forth in SEQ ID NOs: 25 and 26, respectively) on 4114 maize genomic DNA samples and control maize samples. The expected PCR fragment G (257 bp for the 5' genomic region) and PCR fragment H (283 bp for the 3' genomic region) were generated from both 4114 maize and control maize. These PCR products were cloned and sequenced, and the corresponding products from the 4114 maize and the control maize are identical, thus confirming that the sequences are of maize genomic origin.

#### Example 5

##### Insect Efficacy of Maize Event DP-004114-3

Efficacy data was generated on 4114 maize. Field testing compared 4114 maize in two genetic backgrounds to a negative control (isoline) in the same backgrounds. Efficacy testing included: first generation ECB (ECB1) foliage damage and second generation ECB (ECB2) stalk damage at four locations, WCRW root damage at three locations, and FAW foliar damage at one location. At each location, single-row plots were planted in a randomized complete block with three replications (20 kernels/plotx12 entriesx3 replicates=1 experiment/location). All plants were tissue sampled after emergence to confirm the presence of the event by event-specific PCR. Any negatives were culled and each plot thinned to a target stand of 10-15 evenly spaced plants per plot.

For trials characterizing ECB1 damage, each plant was manually infested with approximately 100 ECB neonate larvae 3 times (300 larvae total) over approximately one week beginning at approximately the V5 growth stage. Approximately three weeks after the last successful infestation, leaf damage ratings (based on a 9-1 visual rating scale where 9 indicates no damage and 1 indicates maximum damage) were taken on 8 consecutive plants per plot (total of 24 plants per genetic background, per entry) and means were calculated for each treatment. First generation ECB foliar feeding results on 4114 maize are shown in Table 9.

US 8,575,434 B2

37

TABLE 9

Efficacy of DP-004114-3 Maize Against First Generation ECB Larvae		
Location	Maize Line	Mean ECB1LF Damage Rating $\pm$ Standard Error <sup>a,b</sup>
York, NE	4114	9.0 $\pm$ 0.05 A
	1507 $\times$ 59122	9.0 $\pm$ 0.08 A
	Negative control	4.4 $\pm$ 0.09 B
Johnston, IA	4114	9.0 $\pm$ 0.00 A
	1507 $\times$ 59122	9.0 $\pm$ 0.00 A
	Negative control	4.5 $\pm$ 0.08 B
Mankato, MN	4114	9.0 $\pm$ 0.02 A
	1507 $\times$ 59122	9.0 $\pm$ 0.03 A
	Negative control	4.7 $\pm$ 0.11 B
Princeton, IL	4114	9.0 $\pm$ 0.00 A
	1507 $\times$ 59122	9.0 $\pm$ 0.00 A
	Negative control	5.5 $\pm$ 0.17 B

<sup>a</sup>Damage ratings on individual plants were determined using the following visual rating scale: 9. No visible leaf injury or a small amount of pin or fine shot-hole type injury on a few leaves. 8. Small amount of shot-hole type lesions on a few leaves. 7. Shot-hole injury common on several leaves. 6. Several leaves with shot-hole and elongated lesions (Lesions <0.5" in length). 5. Several leaves with elongated lesions (Lesions 0.5" to 1.0" in length). 4. Several leaves with elongated lesions (Lesions >1.0" in length). 3. Long lesions (>1.0") common on about one-half the leaves. 2. Long lesions (>1.0") common on about two-thirds the leaves. 1. Most of the leaves with long lesions.

<sup>b</sup>Within a location, means with the same letter are not significantly different (Fisher's Protected LSD test,  $P > 0.05$ ).

For trials characterizing ECB2 damage, the same plants infested above for ECB1 were manually infested again later in the growing season with approximately 100 ECB neonate larvae (300 larvae total) per plant 3 times over approximately one week beginning at the R1 growth stage, when approximately 50% of the plants were shedding pollen. At approximately 50-60 days after the last infestation, stalks of 8 consecutive plants per plot (total of 24 plants per genetic background, per entry) were split from the top of the 4th internode above the primary ear to the base of the plant. The total length of ECB stalk tunneling (ECBXCM) was then measured in centimeters and recorded for each plant. Tunnels 1 cm or less were considered entrance holes (larvae was not able to establish in the stalk) and were not included in the total cm of tunneling. Means (total cm of tunneling) were calculated for each treatment. The ECB2 stalk feeding results for 4114 maize are shown in Table 10.

TABLE 10

Efficacy of DP-004114-3 Maize Against Second Generation ECB Larvae		
Location	Maize Line	Mean ECB $\times$ CM (tunnel length, cm) $\pm$ Standard Error <sup>b</sup>
York, NE	4114	0.9 $\pm$ 0.27 B
	1507 $\times$ 59122	0.4 $\pm$ 0.12 B
	Negative control	22.6 $\pm$ 1.83 A
Mankato, MN	4114	1.3 $\pm$ 0.30 B
	1507 $\times$ 59122	0.7 $\pm$ 0.18 B
	Negative control	31.3 $\pm$ 2.19 A
Johnston, IA	4114	1.1 $\pm$ 0.26 B
	1507 $\times$ 59122	0.3 $\pm$ 0.11 B
	Negative control	33.0 $\pm$ 2.51 A
Princeton, IL	4114	0.8 $\pm$ 0.22 B
	1507 $\times$ 59122	0.1 $\pm$ 0.07 B
	Negative control	10.0 $\pm$ 0.94 A

<sup>b</sup>Within a location, means with the same letter are not significantly different (Fisher's Protected LSD test,  $P > 0.05$ ).

Root damage caused by WCRW was also investigated. Plants at approximately the V2 growth stage were manually infested with approximately 500 WCRW eggs applied into the soil on each side of the plant (1,000 eggs/plant total).

38

Additionally, plots were planted in fields that had a high probability of containing a natural infestation of WCRW. Plant roots were evaluated at approximately the R2 growth stage. Five consecutive plants per plot (total 45 plants per genetic background, per entry) were removed from the plot and washed with pressurized water. The root damage was rated using the 0-3 node injury scale (CRWNIS) (Oleson, et al. (2005) *J. Econ. Entomol.* 98(1):1-8) and means were calculated for each treatment. Mean root damage ratings from WCRW feeding are shown in Table 11.

TABLE 11

Efficacy of DP-004114-3 Maize Against WCR Larvae		
Location	Maize Line	Mean CRWNIS score $\pm$ Standard Error <sup>b,c</sup>
Johnston, IA	4114	0.1 $\pm$ 0.01 B
	1507 $\times$ 59122	0.1 $\pm$ 0.02 B
	Negative Control	0.5 $\pm$ 0.09 A
Mankato, MN	4114	0.1 $\pm$ 0.02 B
	1507 $\times$ 59122	0.1 $\pm$ 0.01 B
	Negative Control	1.1 $\pm$ 0.11 A
Rochelle, IL	4114	0.3 $\pm$ 0.04 B
	1507 $\times$ 59122	0.1 $\pm$ 0.01 B
	Negative Control	1.3 $\pm$ 0.18 A

<sup>b</sup>Damage ratings on individual plant root masses were determined using 0-3 Node Injury Scale (Oleson et al. 2005, *supra*).

<sup>c</sup>Within a location, means with the same letter are not significantly different (Fisher's Protected LSD test,  $P > 0.05$ ).

For the FAW efficacy testing, individual plants were manually infested with approximately 75 neonates at approximately the V5 growth stage. Leaves were scored for damage on 8 consecutive plants per plot (total of 24 plants per genetic background, per entry) (FAWLF based on a 9-1 visual rating scale where 9 indicates no damage and 1 indicates maximum damage approximately two weeks after the last successful inoculation and means were calculated for each treatment. Mean damage ratings characterizing FAW foliar feeding on DP-004114-3 are shown in Table 12.

TABLE 12

Efficacy of DP-004114-3 Maize Against FAW Larvae		
Location	Maize Line	Mean FAWLF Damage Rating $\pm$ Standard Error <sup>a,b</sup>
Johnston, IA	4114	8.9 $\pm$ 0.06 BC
	1507 $\times$ 59122	9.0 $\pm$ 0.00 A
	Negative control	2.1 $\pm$ 0.08 D

<sup>a</sup>Damage ratings on individual plants were determined using the following visual rating scale: 9. No damage to pinhole lesions present on whorl leaves. 8. Pinholes and small circular lesions present on whorl leaves. 7. Small circular lesions and a few small elongated (rectangular shaped) lesions up to 0.5" in length present on whorl and furl leaves. 6. Several small elongated lesions 0.5" to 1" in length on a few whorl and furl leaves. 5. Several large elongated lesions greater than 1" in length present on a few whorl and furl leaves and/or a few small to mid-sized uniform to irregular shaped holes (basement membrane consumed) in whorl and furl leaves. 4. Several large elongated lesions present on several whorl and furl leaves and/or several large uniform to irregular shaped holes in whorl and furl leaves. 3. Many elongated lesions of all sizes present on several whorl leaves plus several large uniform to irregular shaped holes in whorl and furl leaves. 2. Many elongated lesions of all sizes present on most whorl and furl leaves plus many mid to large-sized uniform to irregular shaped holes in whorl and furl leaves. 1. Whorl and furl leaves almost totally destroyed.

<sup>b</sup>Within a location, means with the same letter are not significantly different (Fisher's Protected LSD test,  $P > 0.05$ ).

In addition to field efficacy studies, 4114 maize was evaluated in the lab-based sub-lethal seedling assay (SSA) (U.S. Publication No. 2006/0104904 the contents of which is hereby incorporated by reference). The SSA allowed for a comparison of the efficacy of 4114 maize to an unprotected control (near isoline) without the confounding effects of the field environment. The SSA technique involves exposing a population of neonate WCRW to maize seedlings containing either one of the 4114 maize events or non-transgenic (nega-



US 8,575,434 B2

39

tive control) maize seedlings. Larvae were exposed for a period of 17 days from the date of initial egg hatch. The experimental unit for the SSA was a single plastic container with dimensions of 23×30×10 cm (Pactiv Corp., Lake Forest, Ill.). Entries were arranged in a randomized complete block with 3 replications per entry. For each entry, SSA setup involved placing 115 kernels into each container with 225 mL of a 1% thiophanate-methyl fungicide solution and 1000 mL of Metro-Mix 200 plant growth media (Scotts-Sierra Horticultural Products Company, Marysville, Ohio). Immediately after adding the Metro-Mix, WCRW eggs were infested onto the surface of each container at a rate of 1,000 eggs per container. WCRW eggs were pre-incubated at 25° C. so that initial egg hatch was timed to occur 5-7 days after container setup. Infested containers were held in a walk-in environmental chamber with settings of 25° C., 65% relative humidity, and 14:10 light:dark cycle. Larvae were extracted from the containers 17 days post-egg hatch using a Burlese funnel system. A random subsample of 30 larvae per container were selected and their head capsules measured under a dissecting microscope to categorize each into 1 of 3 instars. Data collected includes the age structure of the larval population determined from the number of larvae in each of three potential instars. Histograms that graphically displayed the age distribution of larvae for each entry were plotted and visually compared as shown in FIG. 4.

The pest spectrum for 4114 maize is provided in Table 13.

TABLE 13

Insect Pests That Are Controlled or Suppressed by DP-004114-3 Maize Expressing Cry1F, Cry34Ab1, and Cry35Ab1		
Scientific Name	Common Name	Insect Order
<i>Ostrinia nubilalis</i>	European corn borer (ECB)	Lepidoptera
<i>Helioverpa zea</i>	Corn earworm (CEW)	Lepidoptera
<i>Spodoptera frugiperda</i>	Fall armyworm (FAW)	Lepidoptera
<i>Diatraea grandiosella</i>	Southwestern corn borer (SWCB)	Lepidoptera
<i>Richia albicosta</i>	Western bean cutworm (WBCW)	Lepidoptera
<i>Agrotis ipsilon</i>	Black cutworm (BCW)	Lepidoptera
<i>Elasmopalpus lignosellus</i>	Lesser corn stalk borer (LCSB)	Lepidoptera
<i>Diatraea crambidosella</i>	Southern corn stalk borer (SCSB)	Lepidoptera
<i>Diabrotica virgifera virgifera</i>	Western corn rootworm (WCRW)	Coleoptera
<i>Diabrotica virgifera zea</i>	Mexican corn rootworm (MCR)	Coleoptera
<i>Diabrotica berberis</i>	Northern corn rootworm (NCR)	Coleoptera
<i>Diatraea saccharalis</i>	Sugarcane borer (SCB)	Coleoptera

## Example 6

## Protein Expression and Concentration

## Generation of Plant Material

4114 maize from the PHNAR×BC3F3 generation was grown in five locations in the United States and Canada. Each site employed a randomized complete block design containing four blocks, with each block separated by a buffer distance of at least 36 inches (0.9 m). Each entry was planted in 2-row plots bordered on each side by 1 row of border seed. Leaf Tissue Collection and Processing

One leaf tissue sample was collected in each block at the V9 stage. All samples were collected from impartially selected, healthy, representative plants for each event. Each leaf sample was obtained by selecting the youngest leaf that had emerged at least 8 inches (20 cm, visible tissue) from the whorl. If this leaf was damaged or otherwise unhealthy, the

40

next leaf below it was sampled. The leaf was pruned (cut) from the plant approximately 8 inches (20 cm) from the leaf tip. The leaf sample (including midrib) was cut into ≤1 inch (2.5 cm) pieces and placed in a 50-ml sample vial. The samples were then placed on dry ice until transferred to a freezer (≤−10° C.). Samples were shipped frozen and stored at ≤−10° C. upon arrival. All tissue samples were lyophilized, under vacuum, until dry. The lyophilized leaf samples were finely homogenized in preparation for analysis. Samples were stored frozen between processing steps.

## Protein Concentration Determinations

Concentrations of the Cry1F, Cry34Ab1, Cry35Ab1, and PAT proteins were determined using specific quantitative ELISA methods.

## Protein Extraction

Aliquots of processed leaf tissue samples were weighed into 1.2 mL tubes at the target weight of 10 mg. Each sample analyzed for Cry1F, Cry34Ab1, Cry35Ab1, and PAT protein concentrations was extracted in 0.6 mL of chilled PBST (Phosphate Buffered Saline plus Tween-20). Following centrifugation, supernatants were removed, diluted, and analyzed.

## Determination of Cry1F, Cry34Ab1 and PAT Protein Concentration

The Cry1F, Cry34Ab1 and PAT ELISA kits employed were obtained from EnviroLogix, Inc. (Portland, Me.), and the Cry35Ab1 ELISA kit employed was obtained from Acadia BioScience, LLC (Portland, Me.). The ELISA method for each of these four proteins utilized a sequential “sandwich” format to determine the concentration of the protein in sample extracts. Standards (analyzed in triplicate wells) and diluted sample extracts (analyzed in duplicate wells) were incubated in plate pre-coated with an antibody specific to a single protein chosen from Cry1F, Cry34Ab1, Cry35Ab1 or PAT. Following incubation, unbound substances were washed from the plate. A different specific antibody for the respective selected protein, conjugated to the enzyme horseradish peroxidase (HRP), was added to the plate and incubated. Then, unbound substances were washed from the plate leaving the bound protein “sandwiched” between the antibody coated on the plate and the antibody-HRP conjugate. Detection of the bound antibody-protein complex was accomplished by the addition of substrate, which generated a colored product in the presence of HRP. The reaction was stopped with an acid solution and the optical density (OD) of each well was determined using plate reader. An average of the results from duplicate wells was used to determine the concentration of the Cry1F, Cry34Ab1, Cry35Ab1 or PAT protein in ng/mg sample dry weight.

## Calculations for Determining Protein Concentrations

SoftMax® Pro software was used to perform the calculations required to convert the OD values obtained by the plate reader to protein concentrations.

## 1. Standard Curve

A standard curve was included on each ELISA plate. The equation for the standard curve was generated by the software, which used a quadratic fit to relate the mean OD values obtained for the standards to the respective standard concentration (ng/mL). The quadratic regression equation was applied as follows:

$$y=Cx^2+Bx+A$$

where x=known standard concentration and y=respectively mean absorbance value (OD).

US 8,575,434 B2

41

## 2. Sample Concentration

Interpolation of the sample concentration (ng/ml) was accomplished by solving for x in the above equation using values for A, B, and C determined by the standard curve.

$$\text{Sample Concentration (ng/mL)} = \frac{-B + \sqrt{B^2 - 4C(A - \text{sampleOD})}}{2C}$$

e.g. Curve Parameters: A=0.0476, B=0.4556, C=-0.01910, and sample OD=1.438

$$\begin{aligned} \text{Sample Concentration} &= \frac{-0.4556 + \sqrt{0.4556^2 - 4(-0.01910)(0.0476 - 1.438)}}{2(-0.01910)} \\ &= 3.6 \text{ ng/mL} \end{aligned}$$

Sample concentration values were adjusted for the dilution factor expressed as 1:N

Adjusted Concentration=Sample Concentration×Dilution Factor

e.g. Sample Concentration=3.6 ng/mL and Dilution Factor=1:10

Adjusted Concentration=3.6 ng/mL×10=36 ng/mL

Adjusted sample concentration values were converted from ng/mL to ng/mg sample weight as follows:

ng/mg Sample Weight=ng/mL×Extraction Volume (mL)/Sample Weight (mg)

e.g. Concentration=36 ng/mL, Extraction Volume=0.60 mL, and

Sample Weight=10.0 mg

ng/mg Sample Weight=36 ng/mg×0.60 mL/10.0 mg=2.2 ng/mg

## 3. Lower Limit of Quantitation (LLOQ)

The LLOQ, in ng/mg sample weight, was calculated as follows:

42

$$LLOQ = \frac{\text{Reportable Assay LLOQ} \times \text{Extraction Volume}}{\text{Sample Target Weight}}$$

e.g. for PAT in leaf: reportable assay LLOQ=2.3 ng/mL, extraction volume=0.6 mL, and sample target weight=10 mg

$$LLOQ = \frac{2.3 \text{ ng/mL} \times 0.6 \text{ mL}}{10 \text{ mg}} = 0.14 \text{ ng/mg sample weight}$$

## Results

The proteins Cry1F, Cry34Ab1, Cry35Ab1, and PAT were detected in V9 leaf tissue of 4114 maize at the concentrations set forth in Table 14 below.

TABLE 14

Protein Concentrations in 4114 Maize				
Protein concentration in ng/mg dry weight*				
	Cry1F	Cry34Ab1	Cry35Ab1	PAT
Mean ± SD	9.7 ± 2.5	26 ± 3.1	33 ± 3.1	9.8 ± 3.3
Range	5.3-14	22-31	28-39	4.8-15

\*The LLOQ for Cry1F and PAT was 0.14 ng/mg Dry Weight; the LLOQ for Cry34Ab1 and Cry35Ab1 were 0.16 ng/mg Dry Weight

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 28

<210> SEQ ID NO 1

<211> LENGTH: 605

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<220> FEATURE:

<221> NAME/KEY: PEPTIDE

<222> LOCATION: (0) ... (0)

<223> OTHER INFORMATION: Cry1F

<400> SEQUENCE: 1

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20 25 30

Pro Leu Asp Ile Ser Leu Ser Leu Thr Arg Phe Leu Leu Ser Glu Phe  
35 40 45

Val Pro Gly Val Gly Val Ala Phe Gly Leu Phe Asp Leu Ile Trp Gly  
50 55 60

Phe Ile Thr Pro Ser Asp Trp Ser Leu Phe Leu Leu Gln Ile Glu Gln

US 8,575,434 B2

43

44

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65	70	75	80
Leu Ile Glu Gln Arg 85	Ile Glu Thr Leu Glu Arg 90	Asn Arg Ala Ile Thr 95	
Thr Leu Arg Gly Leu Ala Asp 100	Ser Tyr Glu Ile Tyr Ile Glu Ala Leu 105		
Arg Glu Trp Glu Ala Asn Pro 115	Asn Asn Ala Gln Leu Arg Glu Asp Val 120		
Arg Ile Arg Phe Ala Asn Thr 130	Asp Asp Ala Leu Ile Thr Ala Ile Asn 135		
Asn Phe Thr Leu Thr Ser Phe Glu Ile Pro 145	Leu Leu Ser Val Tyr Val 150		
Gln Ala Ala Asn Leu His Leu Ser Leu Leu Arg Asp 165	Ala Val Ser Phe 170		
Gly Gln Gly Trp Gly Leu Asp Ile Ala Thr Val Asn Asn His Tyr Asn 180			
Arg Leu Ile Asn Leu Ile His Arg Tyr Thr Lys His Cys Leu Asp Thr 195			
Tyr Asn Gln Gly Leu Glu Asn Leu Arg Gly Thr Asn Thr Arg Gln Trp 210			
Ala Arg Phe Asn Gln Phe Arg Arg Asp Leu Thr Leu Thr Val Leu Asp 225			
Ile Val Ala Leu Phe Pro Asn Tyr Asp Val Arg Thr Tyr Pro Ile Gln 245			
Thr Ser Ser Gln Leu Thr Arg Glu Ile Tyr Thr Ser Ser Val Ile Glu 260			
Asp Ser Pro Val Ser Ala Asn Ile Pro Asn Gly Phe Asn Arg Ala Glu 275			
Phe Gly Val Arg Pro Pro His Leu Met Asp Phe Met Asn Ser Leu Phe 290			
Val Thr Ala Glu Thr Val Arg Ser Gln Thr Val Trp Gly Gly His Leu 305			
Val Ser Ser Arg Asn Thr Ala Gly Asn Arg Ile Asn Phe Pro Ser Tyr 325			
Gly Val Phe Asn Pro Gly Gly Ala Ile Trp Ile Ala Asp Glu Asp Pro 340			
Arg Pro Phe Tyr Arg Thr Leu Ser Asp Pro Val Phe Val Arg Gly Gly 355			
Phe Gly Asn Pro His Tyr Val Leu Gly Leu Arg Gly Val Ala Phe Gln 370			
Gln Thr Gly Thr Asn His Thr Arg Thr Phe Arg Asn Ser Gly Thr Ile 385			
Asp Ser Leu Asp Glu Ile Pro Pro Gln Asp Asn Ser Gly Ala Pro Trp 405			
Asn Asp Tyr Ser His Val Leu Asn His Val Thr Phe Val Arg Trp Pro 420			
Gly Glu Ile Ser Gly Ser Asp Ser Trp Arg Ala Pro Met Phe Ser Trp 435			
Thr His Arg Ser Ala Thr Pro Thr Asn Thr Ile Asp Pro Glu Arg Ile 450			
Thr Gln Ile Pro Leu Val Lys Ala His Thr Leu Gln Ser Gly Thr Thr 465			
Val Val Arg Gly Pro Gly Phe Thr Gly Gly Asp Ile Leu Arg Arg Thr 485			

US 8,575,434 B2

45

46

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Ser Gly Gly Pro Phe Ala Tyr Thr Ile Val Asn Ile Asn Gly Gln Leu  
500 505 510

Pro Gln Arg Tyr Arg Ala Arg Ile Arg Tyr Ala Ser Thr Thr Asn Leu  
515 520 525

Arg Ile Tyr Val Thr Val Ala Gly Glu Arg Ile Phe Ala Gly Gln Phe  
530 535 540

Asn Lys Thr Met Asp Thr Gly Asp Pro Leu Thr Phe Gln Ser Phe Ser  
545 550 555 560

Tyr Ala Thr Ile Asn Thr Ala Phe Thr Phe Pro Met Ser Gln Ser Ser  
565 570 575

Phe Thr Val Gly Ala Asp Thr Phe Ser Ser Gly Asn Glu Val Tyr Ile  
580 585 590

Asp Arg Phe Glu Leu Ile Pro Val Thr Ala Thr Leu Glu  
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 <220> FEATURE:  
 <221> NAME/KEY: PEPTIDE  
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 <223> OTHER INFORMATION: Cry34Ab1

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Thr Ser Pro Thr Asn Val Ala Asn Asp Gln Ile Lys Thr Phe Val Ala  
35 40 45

Glu Ser Asn Gly Phe Met Thr Gly Thr Glu Gly Thr Ile Tyr Tyr Ser  
50 55 60

Ile Asn Gly Glu Ala Glu Ile Ser Leu Tyr Phe Asp Asn Pro Phe Ala  
65 70 75 80

Gly Ser Asn Lys Tyr Asp Gly His Ser Asn Lys Ser Gln Tyr Glu Ile  
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Met Asn Lys Asn Asp Asp Asp Ile Asp Asp Tyr Asn Leu Lys Trp Phe  
35 40 45

Leu Phe Pro Ile Asp Asp Asp Gln Tyr Ile Ile Thr Ser Tyr Ala Ala  
50 55 60

US 8,575,434 B2

47

48

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Asn Asn Cys Lys Val Trp Asn Val Asn Asn Asp Lys Ile Asn Val Ser  
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 Thr Tyr Ser Ser Thr Asn Ser Ile Gln Lys Trp Gln Ile Lys Ala Asn  
 85 90 95  
 Gly Ser Ser Tyr Val Ile Gln Ser Asp Asn Gly Lys Val Leu Thr Ala  
 100 105 110  
 Gly Thr Gly Gln Ala Leu Gly Leu Ile Arg Leu Thr Asp Glu Ser Ser  
 115 120 125  
 Asn Asn Pro Asn Gln Gln Trp Asn Leu Thr Ser Val Gln Thr Ile Gln  
 130 135 140  
 Leu Pro Gln Lys Pro Ile Ile Asp Thr Lys Leu Lys Asp Tyr Pro Lys  
 145 150 155 160  
 Tyr Ser Pro Thr Gly Asn Ile Asp Asn Gly Thr Ser Pro Gln Leu Met  
 165 170 175  
 Gly Trp Thr Leu Val Pro Cys Ile Met Val Asn Asp Pro Asn Ile Asp  
 180 185 190  
 Lys Asn Thr Gln Ile Lys Thr Thr Pro Tyr Tyr Ile Leu Lys Lys Tyr  
 195 200 205  
 Gln Tyr Trp Gln Arg Ala Val Gly Ser Asn Val Ala Leu Arg Pro His  
 210 215 220  
 Glu Lys Lys Ser Tyr Thr Tyr Glu Trp Gly Thr Glu Ile Asp Gln Lys  
 225 230 235 240  
 Thr Thr Ile Ile Asn Thr Leu Gly Phe Gln Ile Asn Ile Asp Ser Gly  
 245 250 255  
 Met Lys Phe Asp Ile Pro Glu Val Gly Gly Gly Thr Asp Glu Ile Lys  
 260 265 270  
 Thr Gln Leu Asn Glu Glu Leu Lys Ile Glu Tyr Ser His Glu Thr Lys  
 275 280 285  
 Ile Met Glu Lys Tyr Gln Glu Gln Ser Glu Ile Asp Asn Pro Thr Asp  
 290 295 300  
 Gln Ser Met Asn Ser Ile Gly Phe Leu Thr Ile Thr Ser Leu Glu Leu  
 305 310 315 320  
 Tyr Arg Tyr Asn Gly Ser Glu Ile Arg Ile Met Gln Ile Gln Thr Ser  
 325 330 335  
 Asp Asn Asp Thr Tyr Asn Val Thr Ser Tyr Pro Asn His Gln Gln Ala  
 340 345 350  
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 <223> OTHER INFORMATION: Phosphinothricin acetyl transferase protein  
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US 8,575,434 B2

49

50

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Glu Gly Val Val Ala Gly Ile Ala Tyr Ala Gly Pro Trp Lys Ala Arg		
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Asn Ala Tyr Asp Trp Thr Val Glu Ser Thr Val Tyr Val Ser His Arg		
85	90	95
His Gln Arg Leu Gly Leu Gly Ser Thr Leu Tyr Thr His Leu Leu Lys		
100	105	110
Ser Met Glu Ala Gln Gly Phe Lys Ser Val Val Ala Val Ile Gly Leu		
115	120	125
Pro Asn Asp Pro Ser Val Arg Leu His Glu Ala Leu Gly Tyr Thr Ala		
130	135	140
Arg Gly Thr Leu Arg Ala Ala Gly Tyr Lys His Gly Gly Trp His Asp		
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Val Gly Phe Trp Gln Arg Asp Phe Glu Leu Pro Ala Pro Pro Arg Pro		
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Val Arg Pro Val Thr Gln Ile		
180		

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&lt;211&gt; LENGTH: 11978

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Complete sequence of the T-DNA region of Plasmid PHP27118

&lt;400&gt; SEQUENCE: 5

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tgcagcgtga cccggtcgtg cccctctcta gagataatga gcattgcatg tctaagttat	180
aaaaaattac cacatatttt tttgtcaca cttgtttgaa gtgcagttaa tctatcttta	240
tacatatatt taaactttac tctacgaata atataatcta tagtactaca ataataatcag	300
tggttttagag aatcatataa atgaacagtt agacatggtc taaaggacaa ttgagtattt	360
tgacaacagg actctacagt tttatctttt tagtgtgcat gtgttctcct ttttttttgc	420
aaatagcttc acctatataa tacttcatcc attttattag tacatccatt tagggtttag	480
ggttaatggt ttttatagac taattttttt agtacatcta ttttattcta ttttagcctc	540
taaaattaaga aaactaaaac tctatttttag tttttttatt taataattta gatataaaat	600
agaataaaat aaagtgacta aaaattaaac aaataccctt taagaaatta aaaaaactaa	660
ggaaacattt ttcttggttc gagtagataa tgccagcctg ttaaagcgccg tcgacgagtc	720
taacggacac caaccagcga accagcagcg tcgctcgagg ccaagcgaag cagacggcac	780
ggcatctctg tcgctgcctc tggaccctc tcgagagttc cgctccaccg ttggacttgc	840
tccgctgtcg gcacccagaa attgcgtggc ggagcggcag acgtgagccg gcacggcagg	900
cggcctcctc ctctctctac ggcaccggca gctacggggg attcctttcc caccgctcct	960
tcgctttccc ttctctgccc gccgtaataa atagacaccc cctccacacc ctctttcccc	1020
aacctcgtgt tggtcggagc gcacacacac acaaccagat ctccccaaa tccaccgctc	1080
ggcacctccg cttcaaggta cgcgctcgt cctccccccc cccctctctc taccttctct	1140
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US 8,575,434 B2

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US 8,575,434 B2

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US 8,575,434 B2

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US 8,575,434 B2

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US 8,575,434 B2

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&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: The complete sequence of the insert and flanking regions of event DP-004114-3

&lt;400&gt; SEQUENCE: 6

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US 8,575,434 B2

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US 8,575,434 B2

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US 8,575,434 B2

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US 8,575,434 B2

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US 8,575,434 B2

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US 8,575,434 B2

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US 8,575,434 B2

73

74

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US 8,575,434 B2

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76

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US 8,575,434 B2

77

78

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US 8,575,434 B2

79

80

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US 8,575,434 B2

81

82

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US 8,575,434 B2

83

What is claimed is:

1. A DNA construct comprising: a first, second, third and fourth expression cassette, wherein said first expression cassette in operable linkage comprises:

- (a) a maize ubiquitin promoter;
  - (b) a 5' untranslated exon of a maize ubiquitin gene;
  - (c) a maize ubiquitin first intron;
  - (d) a Cry1F encoding DNA molecule; and
  - (e) a poly(A) addition signal from ORF 25 terminator;
- said second expression cassette in operable linkage comprises:

- (1) a maize ubiquitin promoter;
- (2) a 5' untranslated exon of a maize ubiquitin gene;
- (3) a maize ubiquitin first intron;
- (4) a Cry34AbI encoding DNA molecule; and
- (5) a PinII transcriptional terminator;

said third expression cassette in operable linkage comprises:

- (i) a wheat peroxidase promoter;
- (ii) a Cry35AbI encoding DNA molecule; and
- (iii) a PinII transcriptional terminator; and

said fourth expression cassette in operable linkage comprises:

- (a) a CaMV 35S promoter;
- (b) a pat encoding DNA molecule; and
- (c) a 3' transcriptional terminator from CaMV 35S;

wherein the four cassettes are flanked by SEQ ID NO: 27 at the 5' end and SEQ ID NO: 28 at the 3' end.

2. A plant comprising the DNA construct of claim 1.

3. A plant of claim 2, wherein said plant is a corn plant.

4. A plant comprising the sequence set forth in SEQ ID NO: 6.

5. A corn plant comprising the genotype of the corn event DP-004114-3 deposited with American Type Culture Collection (ATCC) under Accession No. PTA-11506, wherein said genotype comprises the DNA construct of claim 1.

6. A corn plant comprising the genotype of the corn event DP-004114-3, wherein a representative sample of seed of said corn event has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

7. A plant parts of the plant comprising the corn event of claim 6.

8. A seed comprising corn event DP-004114-3, wherein said seed comprises the DNA construct of claim 1, wherein a representative sample of corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

9. A corn plant, or part thereof, grown from the seed of claim 8.

10. A transgenic seed produced from the corn plant of claim 9, wherein the seed comprises corn event DP-004114-3.

11. A transgenic corn plant, or part thereof, grown from the seed of claim 10.

12. An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 6; SEQ ID NO: 27; SEQ ID NO: 28, and full length complements thereof.

13. An amplicon comprising the nucleic acid sequence selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28 and full length complements thereof.

14. A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic

84

acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

15. The biological sample of claim 14, wherein said biological sample comprise plant, tissue, or seed of transgenic corn event DP-004114-3.

16. The biological sample of claim 15, wherein said biological sample is a DNA sample extracted from the transgenic corn plant event DP-004114-3, and wherein said DNA sample comprises one or more of the nucleotide sequences selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28, and the complement thereof.

17. The biological sample of claim 16, wherein said biological sample is selected from the group consisting of corn flour, corn meal, and cereals manufactured in whole or in part to contain corn by-products.

18. An extract derived from corn event DP-004114-3 plant, tissue, or seed and comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28 or the complement thereof, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

19. The extract of claim 18, wherein said nucleotide sequence is detectable in said extract using a nucleic acid amplification or nucleic acid hybridization method.

20. The extract of claim 19, further comprising a composition selected from the group consisting of corn flour, corn meal, corn syrup, corn oil, corn starch, and cereals manufactured in whole or in part to contain corn by-products, wherein said composition comprises a detectable amount of said nucleotide sequence.

21. A method of producing hybrid corn seeds comprising: (a) planting seeds of a first inbred corn line comprising the DNA construct of claim 1 and seeds of a second inbred line having a genotype different from the first inbred corn line;

(b) cultivating corn plants resulting from said planting until time of flowering;

(c) emasculating said flowers of plants of one of the corn inbred lines;

(d) sexually crossing the two different inbred lines with each other; and

(e) harvesting the hybrid seed produced thereby.

22. The method according to claim 21, wherein the plants of the first inbred corn line are the female parents.

23. The method according to claim 21, wherein the plants of first inbred corn line are the male parents.

24. A method for producing a corn plant resistant to lepidopteran pests comprising:

(a) sexually crossing a first parent corn plant with a second parent corn plant, wherein said first or second parent corn plant comprises event DP-004114-3 DNA, deposited under Accession No. PTA-11506 with American Type Culture Collection (ATCC), thereby producing a plurality of first generation progeny plants;

(b) selecting a first generation progeny plant that is resistant to lepidopteran insect infestation;

(c) selfing the first generation progeny plant, thereby producing a plurality of second generation progeny plants; and

(d) selecting from the second generation progeny plants, a plant that is resistant to lepidopteran pests; wherein the second generation progeny plants comprise the DNA construct according to claim 1.

US 8,575,434 B2

85

25. The method of claim 21 further comprising backcross-  
ing the second generation progeny plant of step (d) that com-  
prises corn event DP-004114-3 DNA, deposited under Acces-  
sion No. PTA-11506 with American Type Culture Collection  
(ATCC), to the parent plant that lacks the corn event 5  
DP-004114-3 DNA, thereby producing a backcross progeny  
plant that is resistant to at least western corn rootworm.

26. A method for producing a corn plant resistant to at least  
corn rootworm, said method comprising:

- (a) sexually crossing a first parent corn plant with a second 10  
parent corn plant, wherein said first or second parent  
corn plant is a corn event DP-004114-3, deposited under  
Accession No. PTA-11506 with American Type Culture  
Collection (ATCC), plant, thereby producing a plurality  
of first generation progeny plants; 15
- (b) selecting a first generation progeny plant that is resis-  
tant to at least corn rootworm infestation;
- (c) backcrossing the first generation progeny plant of step  
(b) with the parent plant that lacks corn event  
DP-004114-3 DNA, thereby producing a plurality of 20  
backcross progeny plants; and
- (d) selecting from the backcross progeny plants, a plant  
that is resistant to at least corn rootworm infestation;  
wherein the selected backcross progeny plant of step (d)  
comprises SEQ ID NO: 6. 25

27. The method according to claim 21, wherein the plants  
of the first inbred corn line are the female parents or male  
parents.

28. Hybrid seed produced by the method of claim 21.

\* \* \* \* \*

30

86



# **Exhibit C**

(12) **United States Patent**  
**Bing et al.**

(10) **Patent No.:** **US 7,956,246 B2**  
(45) **Date of Patent:** **\*Jun. 7, 2011**

(54) **CORN EVENT DAS-59122-7 AND METHODS FOR DETECTION THEREOF**

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This patent is subject to a terminal disclaimer.

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**A01H 5/10** (2006.01)

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See application file for complete search history.

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(57) **ABSTRACT**

The invention provides DNA compositions that relate to transgenic insect resistant maize plants. Also provided are assays for detecting the presence of the maize DAS-59122-7 event based on the DNA sequence of the recombinant construct inserted into the maize genome and the DNA sequences flanking the insertion site. Kits and conditions useful in conducting the assays are provided.

**14 Claims, 7 Drawing Sheets**

## U.S. Patent

Jun. 7, 2011

Sheet 1 of 7

US 7,956,246 B2

FIG. 1 (Sheet 1 of 5)

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1 CTGAGCGCAC AACAGCGAGT CGCATGGCAC CGGACGACAT GAGCGAGATT
51 TAGATCCGAG GGTGCGGACA TGGGGCAACC TGCGCAGCTA ACGCAGCGAT
101 CCACACGACC ACCAACGAAG CCAAGCCCGG GCACGTCCCC AGGCAGGTTG
151 GGCCCTGGTT CCACCAGCGG ATGCATGCAG TGAAGCGGGG ACGGAGAGAC
201 AAGCCGAGGG CGCGGGTGGG AATGGCGTCC GGGAGGACGA GTGGAGGAGA
251 AGAATCTAGA GGCATCGAGA TTCGAGAAGC CGACGGAGAC AAGATTCCGTG
301 TGGGGGGAGA CAAACCGCGG GGCTGAGCGC CGTTGATATG GGATCAGACG
351 GTGTGGATAA AAAAAGTGAC GTTGATAGAA CGTCTGGCCA GTGAAAAAAC
401 AAAACAACCTC CAACAAAATA CTTTAAAAGC TCTTATACCC TAAATGTAGG
451 GGATCAAAACA CGTCTCTACA CTATTTAGCA GCGTCCTCTA AATGATCCTC
501 TAAATTTAGA GAACGCTACT AGATTCTCTA TATATAGTTT CTCTAAACGA
551 TCTTTTATCC ATTTAAATAC TTAAATAAC CGGTTTAACA AAATAAAAT
601 ATATACAATA CATTGAGAG TATGACAAAT ACGTATGTAT AAAAATAAAA
651 AATAAAATAA TGTATTAGTC TACTTTGAAT CTTCTTTTCT TCATAATATA
701 ATGATGTATA GCTCTCATGT GCGTTGAGAA AAAAGTTAGA GCTAGACGTT
751 TAATGTGTAG TGACAGTCTT GCACGAAATC TCCCTAATGA GATGAATTAC
801 TGGAGGTTCC ATCAGAAAGT CCCCTGAAAA GAGGCATTTA TTTAGTTTAG
851 TCAGCAATTT CTGGGAACAC AAATATTCTT TTGTTATCAC CACTATTAAA
901 AATCTATGGT TATAACTTAT AATAACATGA AAAAATAATT TAGCATCCCA
951 TATATATAAA AACTGAAGGA AGCCATATAT ACTAACATAA GTTAGGAGAA
1001 ACTAAGAAGG TTGTGCAAAG CTTGCACTGC TCCAAAATAC TGCAAACAAC
1051 CACTCTCCTC TACCAACCAA AGAAACTCAT GTACTCCCTC CGTTCTTTT
1101 TATTTGTGCG ATTTTAGTTF AAAAATGAAC TAGCAGTCGA CAAATATTCTG
1151 AGAACAGATA TAGTATATAC TAACATAACT TAGGAGATAC TAAGAAAGTT
1201 GCGCAGAGCT TCACTGTTC CAAATTACTG CAAAGCCTCT CCCCTCTGCC
1251 AGTACATCTA CGAGATGTTT CAGTTAAACA AAGATTCAGA CAAGTGATGA
1301 GCCACTTCTT GTCATAGATT GTGTGGTCAA CCAACCCATT GATGCCACGG
1351 TTTTGTGCA TCCATGCTTT TGTATTAAAA CATCAGTTAT GTTTACCATG
1401 TCCGATATGC TCTACATAAT GACAATCAAC TTGGTGTTC TATATTATAC
1451 AATGTTAGGA ATTTCAATAG CTACGAACAC TTCAATAGAA GTGCCTTTGT
1501 GGGTCAACCT TAATGTGTTG TTGATGTAAG GAGAAGAATC TTAATTTACT
1551 CTTGCTAAAT TTGAACTACA CAAAACCACT GCACTGAGGA TTGTCTTAAT
1601 AAATTACTGC TCATACACGT TAGCATCTGT TCAGATACTG AGCTAATCCC
1651 TAGGATTAAA GSATTTGTAA AAGATATGCC CAATCATTCA TTTTAGTTAT
1701 TTATTTCTTA GTTATCCACT TGAAGATTTA CATACATTTG AAATAAATTT
1751 CTTAGAGGTA AAGTGAAAAT CAGTTATTTA AATACATTTT AGTTATTTAT
1801 TTTCTTCTTT TTCCTAATTT TTCCTTGAT TTGAAGTCTG AAAAGATAAC
1851 TTIGCCCTTA TACATAFTTT ATCTTCTACG TACGCATCTG AACACGTCT
1901 CTTTGTCCCC TGATCGTGCA GCAATTAGTG CTATGAATCG CGTTTAAGCG
1951 CTGCAAAATC ATGGCTGGGG CTTGCTCCTC GAGTCGTCCT GCTGCTCGAT
2001 GTCACCTCGA GTCCCGCACC GACCTCAGTG CTTGTTCTTG TTGGAGCCAC
2051 CTCTCTCGGA CGATCGCCAA AGACGGATAA GGCCGAAGCC GTCACCTCAG
2101 ACCGCGCTCA TGCGCCGTAG CAGACTCCTA CATAGCAGGG CCAGGGTATG
2151 TGGACCTTTG CAAGTTTAGG ATTTGGAACCA GCGACCAGAA TCCACAAGAT
2201 TGGAGCAAAAC GACCABAAAT TCACAAGGAT TGGCGGCTGA CATTGCCAGC
2251 GCGGGATCGC ATGCGGCGGC GCGGCGCGGG GCGAGCACGG GAGCAGGCGA
2301 CAGTCGAGCT CCATTGGAAC GTAGAAATAC TTAAGGGCAA GGTCTCCAAA
2351 TACTTGAAAA AATAGGAAAA AGAAGAAAAT ACATGAAATG ATATTGAAAT
2401 CAATTGGAAG ATGTTATGAA TCTTGTTTTT GCAAAGCGAA CGATTCAGAT
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## U.S. Patent

Jun. 7, 2011

Sheet 2 of 7

US 7,956,246 B2

FIG. 1 (Sheet 2 of 5)

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2451 GCGAAACTA TGAATCTTTT TGTGTGAAGT CCCAAATATA AAATTTTCTC
2501 GTACTCACCA ACATTGGTGC GCACCTGTGA TTGGCTCATA AAAATTTCTG
2551 GAGGGACGSA AGAAAGAGTG AAGGGATAAG CAAGTAAAAG CGCTCAAACA
2601 CTGATAGTTT AAAC TGAAGG CGGGAAACGA CAATCTGATC ATGAGCGGAG
2651 AATTAAGGGA GTCACGTTAT GACCCCCGCC GATGACGCGG GACAAGCCGT
2701 TTTACGTTTG GAACTGACAG AACCGCAACG TTGAAGGAGC CACTCAGCAA
2751 GCTTACTAGT AGCGCTGTTT AAACGCTCTT CAACTGGAAG AGCGGTACC
2801 CGGACCGAAG CTTGCATGCC TGCAGTGCGG CGTGACCCGG TCGTGCCCTT
2851 CTCTAGAGAT AATGAGCATT GCATGTCTAA GTTATAAAAA ATTACCACAT
2901 ATTTTFTTTG TCACACTTGT TTGAAGTGCA GTTTATCTAT CTTTATACAT
2951 ATATTTTAAAC TTTACTCTAC GAATAATATA ATCTATAGTA CTACAATAAT
3001 ATCAGTGTTT TAGAGAATCA TATAAATGAA CAGTTAGACA TGGTCTAAAG
3051 GACAAFTGAG TATTTTGACA ACAGGACTCT ACAGTTTAT CTTTTTAGTG
3101 TGCATGTGTT CTCTTTTTTT TTTGCAAATA GCTTCACCTA TATAAFACTT
3151 CATCCATTTT ATTAGTACAT CCATTAGGG TTAGGGTTA ATGGTTPPTA
3201 TAGACTAATT TTTTATGATC ATCTATTTTA TTCTATTTTA CCTCTAAAT
3251 TAAGAAAAC TAAACTCTAT TTTAGTTTTT TTATTTAATA ATTTAGATAT
3301 AAAATAGAAT AAAATAAAGT GACTAAAAAT TAAACAAATA CCTTTAAGA
3351 AATTAAGAAA ACTAAGGAAA CATTTTCTT GTTTCGAGTA GATAATGCCA
3401 GCCTGTAAAA CGCCGTCGAC GAGTCTAACG GACACCAACC AGCGAACCAG
3451 CAGCGTCGCG TCGGGCCAAG CGAAGCAGAC GGCACGGCAT CTCTGTCGCT
3501 GCCTCTGGAC CCTCTCGAG AGTTCCGCTC CACCGTTGGA CTGTCTCCGC
3551 TGTCGSCATC CAGAAATTGC GTGGCGGAGC GGCAGACGTG AGCCGGCACC
3601 GCAGGCGGCC TCCTCCTCCT CTCACGGCAC CGGCAGCTAC GGGGGATTCC
3651 TTTCCACCCG CTCCTTCGCT TTCCCTTCTT CGCCCGCCGT AATAAATAGA
3701 CACCCCTCC ACACCTCTT TCCCAACCT CGTGTGTTC GGAGCGCACA
3751 CACACACAAC CAGATCTCCC CCAATCCAC CCGTCGGCAC CTCCGCTCA
3801 AGGTACGCCG CTCGTCTCC CCCCCCCCC CTCTCTACCT TCTCTAGATC
3851 GCGGTTCGGG TCCATGGTTA GGGCCCCGTA GTTCTACTTC TGTTCACTGT
3901 TGTGTTAGAT CCGTGTGTG GTTAGATCCG TGCTGCTAGC GTTCGTACAC
3951 GGATGCGACC GTTACGTGAC ACACGTTCTG ATTGCTAACT TGCCAGTGT
4001 TCTCTTTGGG GAATCCTGGG ATGGCTCTAG CCGTTCCGCA GACGGGATCG
4051 ATTTCAATGAT TTTTFTTGT TCGTTGCATA GGGTTTGGTT TGCCCTTTTC
4101 CTTTATTTCA ATATATGCCG TGCACTTGTT TGTCCGGTCA TCTTTTCATG
4151 CTTTTTTTTG TCTTGGTTGT GATGATGTGG TCTGGTTGGG CGGTCTTCT
4201 AGATCGGAGT AGAATCTGT TTCAAAC TAC TCGGTGGATT TATTAAATTT
4251 GGATCTGTAT GTGTGTGCCA TACATATCA TAGTTACGAA TTGAAGATGA
4301 TGGATGGAAA TATCGATCTA GGATAGGTAT ACATGTTGAT GCGGGTTTA
4351 CTGATGCATA TACAGAGATG CTTTTGTTC GCTTGGTTGT GATGATGTGG
4401 TGTGGTTGGG CGGTCTTCA TCTGTCTAG ATCGGAGTAG AATACTGTTT
4451 CAACTACCT GGTGTATTTA TTAATTTTG AACTGTATGT GTGTGTCATA
4501 CATCTTCATA GTTACGAGTT TAAGATGGAT GGAAATATCG ATGTAGGATA
4551 GGTATACATG TTGATGTGG TTTTACTGAT GCATATACAT GATGGCATAT
4601 GCAGATCTA TTCAATATGCT CTAACCTGA GTACCTATCT ATTATAATAA
4651 ACAAGTATGT TTTAATTTA TTTGATCTT GATATACTTG GATGATGGCA
4701 TATGCAGCAG CTATATGTGG ATTTTTTTAG CCTTGCCTTC ATACGCTATT
4751 TATTTGCTTG GTACTGTTTC TTTTGTGAT GCTCACCCTG TTGTTTGGTG
4801 TTACTTCTGC AGGTGACTC TAGAGGATCC ACACGACACC ATGTCCGCC
4851 GCGAGGTGCA CATCGACGTG AACAACAAGA CCGCCACAC CCTCCAGCTG
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U.S. Patent

Jun. 7, 2011

Sheet 3 of 7

US 7,956,246 B2

FIG. 1 (Sheet 3 of 5)

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4901 GAGGACAAGA CCAAGCTCGA CGGCGGCAGG TGGCGCACCT CCCCAGACAA
4951 CCGTGGCCAAC GACCAGATCA AGACCTTCCT GGCCGAATCC AACGGCTTCA
5001 TGACCGGCAC CGAGGGCACC ATCTACTACT CAATTAATGG CGAGGCCGAG
5051 ATCAGCCTCT ACTTCGACAA CCCGTTCCGC GGCTCCAACA AATACGACGG
5101 CCACTCCAAC AAGTCCCACT ACGAGATCAT CACCCAGGGC GGCTCCGGCA
5151 ACCAGTCCCA CGTGACCTAC ACCATCCAGA CCACCTCCTC CCGCTACGGC
5201 CACAAGTCCT GAGTCATGAG TCATGAGTCA GTTAACCTAG ACTTGTCCTT
5251 CTTCCTGGATT GGCCAACCTA ATTAATGTAT GAAATAAAAG GATGCACACA
5301 TAGTGACATG CTAATCACTA TAATGTGGGC ATCAAAGTTG TGTGTTATGT
5351 GTAATTACTA GTTATCTGAA TAAAAGAGAA AGAGATCATC CATATTTCTT
5401 ATCCTAAATG AATGTCACGT GTCTTTTATA TTCTTTGATG AACCAGATGC
5451 ATTTCAATTA CCAAATCCAT ATACATATAA ATATTAATCA TATATAATTA
5501 ATATCAATTG GGTTAGCAAA ACAAATCTAG TCTAGGTGTG TTTTGCGAAT
5551 GCGGCCCGCG ACCGAATTGG GGATCTGCAT GAAAGAAACT GTCGCACTGC
5601 TGAACCGCAC CTTGTCACTT TCATCGAACA CGACCTGTGC CCAAGATGAC
5651 GGTGCTGCGG TCTAAGTGAG GCTGAATTGC CTTGGACAGA AGCGGACTCC
5701 CTACAATTAG TTAGGCCAAA CGGTGCAATC ATGTGTAGCT CCGGGCTCGG
5751 GCTGTATCGC CATCTGCAAT AGCATCCATG GAGCTCGTTC CATGTAGTTG
5801 GAGATGAACC AATGATCGGG CGTGTGGACG TATGTTTCTG TGTACTCCGA
5851 TAGTAGAGTA CGTGTTAGCT CTTTCATGCT GCAAGTGAAA TTTGTGTGTTG
5901 TTTAATTACC CCTACGTTAG TTGCGGGACA GGAGACACAT CATGAATTTA
5951 AAGGCGATGA TGTCTCTCC TGTAAATGTTA TTCTTTTGAT GTGATGAATC
6001 AAAATGTCAT ATAAAACATT TGTTCCTCTT TAGTTAGGCC TGATCGTAGA
6051 ACGAAATGCT CGTGTAGCGG GGCTACGAGC CTATGACGCA ATAACACTGG
6101 TTTGCCGGCC CGGAGTCGCT TGACAAAAAA AAGCATGTTA AGTTTATTTA
6151 CAATTCAAAA CCTAACATAT TATATTCCTT CAAAGCAGGT TCACGATCAC
6201 ACCTGTACCT AAAAAAACA TGAAGAATAT ATTACTCCAT TATTATGAGA
6251 TGAACCACTT GGCAAGAGTG GTAAGCTATA TAAAAAATG AACATTATTA
6301 CGAGATGTTA TATGCCATTA TATTGATTCT AAGATATATG TTTCTTTCTC
6351 CCACGGGCAC CTAACGGATA CATGATAAGG CCAAGGCAGA TCACGGGAAA
6401 TTATTGCAAT ACATGTTACG CCTATTGCC GGAAAAAAA TGCAGGGCAG
6451 GTGTTGGCCG TAGCGATTTA AGCACTTAAG CTGGAGGTTG CCACACTTGG
6501 ATGCAAGCGT CTGACCCCTT TAAAAACATG GCGGCTTTGT CCGTATCCGT
6551 ATCCCTATC CGACATCTAG CTGCCACAC GACGGGGCTG GGCAGATCGT
6601 GGATGCCGGG TCGACGTCGA TCGTCAGCCA TCATAGACCA ATCGACCATC
6651 TGTATGGAT GCTTGCTAGC TAGACTAGTC AGACATAAAA TTTTGGATACT
6701 TTCTCCCAAC TGGGAGACGG GGACTGATGT GCAGCTGCAC GTGAGCTAAA
6751 TTTTCCCTA TAAATATGCA TGAAATACTG CATTAATCTT CCACAGCCAC
6801 TGCCACAGCC AGATAACAAG TGCAGCTGGT AGCACGCAAC GCATAGCTCT
6851 GGACTTGTAG CTAGGTAGCC AACCGGATCC ACACGACACC ATGCTCGACA
6901 CCAACAAGGT GTACGAGATC AGCAACCACG CCAACGGCCT CTACGCCGCC
6951 ACCTACTCTT CCTCGACGA CTCCGGCGTG TCCCTCATGA ACAAGAACGA
7001 CGACGACATC GACGACTACA ACCTCAAGTG GTTCTCTTTC CCGATCGACG
7051 ACGACCACTA CATCATCACC TCCFACGCCG CCAACAACCTG CAAGGTGTGG
7101 AACGTGAACA ACGACAAGAT TAATGTGTCA ACCTACTCCT CCACCAACTC
7151 CATCCAGAAG TGGCAGATCA AGGCCAACGG CTCTCTCTAC GTGATCCAGT
7201 CCGACAACGG CAAGGTGCTC ACCGCCGGCA CCGGCCAGGC CCTCGGCCCTC
7251 ATCCGCCTCA CCGACGAGTC CTCCAACAAC CCGAACCAGC AATGGAACCT
7301 GACGTCCGTG CAGACCATCC AGCTCCCGCA GAAGCCGATC ATCGACACCA
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U.S. Patent

Jun. 7, 2011

Sheet 4 of 7

US 7,956,246 B2

## FIG. 1 (Sheet 4 of 5)

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7351 AGCTCAAGGA CTACCCGAAG TACTCCCCGA CCGGCAACAT CGACAACGGC
7401 ACCTCCCCGC AGCTCATGGG CTGGACCCTC GTGCCGTGCA TCATGGTGAA
7451 CGACCCGAAC ATCGACAAGA ACACCCAGAT CAAGACCACC CCGTACTACA
7501 TCCTCAAGAA GTACCAGTAC TGGCAGAGGG CCGTGGGCTC CAACGTCGCG
7551 CTCCGCCCGC ACGAGAAGAA GTCCTACACC TACGAGTGGG GCACCGAGAT
7601 CGACCAGAAG ACCACCATCA TCAACACCCT CGGCTTCCAG ATCAACATCG
7651 ACAGCGGCAT GAAGTTCGAC ATCCCGGAGG TGGGCGGCGG TACCGACGAG
7701 ATCAAGACCC AGCTCAACGA GGAGCTCAAG ATCGAGTATT CACATGAGAC
7751 GAAGATCATG GAGAAGTACC AGGAGCAGTC CGAGATCGAC AACCCGACCG
7801 ACCAGTCCAT GAACTCCATC GGCTTCCTCA CCATCACCTC CCTGGAGCTC
7851 TACCGCTACA ACGGCTCCGA GATCCGCATC ATGCAGATCC AGACCTCCGA
7901 CAACGACACC TACAACGTGA CCTCCTACCC GAACCACCAG CAGGCCCTGC
7951 TGCTGCTGAC CAACCACTCC TACGAGGAGG TGGAGGAGAT CACCAACATC
8001 CCGAAGTCCA CCTCAAGAA GCTCAAGAAG TACTACTTCT GAGTCATGAG
8051 TCATGAGTCA GTTAACCTAG ACTTGTCAT CTCTTGATT GGCCAACTTA
8101 ATTAATGTAT GAAATAAAAG GATGCACACA TAGTGACATG CTAATCACTA
8151 TAATGTGGGC ATCAAAGTTG TGTGTTATGT GTAATTACTA GTTATCTGAA
8201 TAAAAGAGAA AGAGATCATC CATATTTCTT ATCCTAAATG AATGTCACGT
8251 GTCTTTATAA TTCTTTGATG AACAGATGC ATTTCAATTA CCAAATCCAT
8301 ATACATATAA ATATTAATCA TATATAATTA ATATCAATTG GGTTAGCAAA
8351 ACAAATCTAG TCTAGGTGTG TTTTGCGAAT TCCCATGGAG TCAAAGATTC
8401 AAATAGAGGA CCTAACAGAA CTCGCCGTAA AGACTGGCGA ACAGTTCATA
8451 CAGAGTCTCT TACGACTCAA TGACAAGAAG AAAATCTTCG TCAACATGGT
8501 GGAGCACGAC ACGCTTGCTT ACTCCAAAAA TATCAAAGAT ACAGTCTCAG
8551 AAGACCAAAG GGCAATTGAG ACTTTTCAAC AAAGGGTAAT ATCCGGAAAC
8601 CTCTCTGGAT TCCATFGCCC AGCTATCTGT CACTTTATTG TGAAGATAGT
8651 GGAAAAGGAA GGTGGCTCCT ACAAATGCCA TCATTGCGAT AAAGGAAAGG
8701 CCATCGTTGA AGATGCCTCT GCCGACAGTG GTCCCAAAGA TGGACCCCCA
8751 CCCACGAGGA CCATCGTGGG AAAAGAAGAC GTTCCAACCA CGTCTTCAAA
8801 GCAAGTGGAT TGATGTGATA TCTCCACTGA CGTAAGGGAT GACGCACAA
8851 CCCACTATCC TTCGCAAGAC CCTTCCTCTA TATAAGGAAG TTCATTTCT
8901 TTGGAGAGGA CAGGGTACCC GGGGATCCAC CATGTCTCCG GAGAGGAGAC
8951 CAGTTGAGAT TAGGCCAGCT ACAGCAGCTG ATATGGCCGC GGTTTGTGAT
9001 ATCGTTAACC ATTACATTGA GACGCTTACA GTGAACTTTA GGACAGAGCC
9051 ACAAACACCA CAAGAGTGGG TTGATGATCT AGAGAGGTTG CAAGATAGAT
9101 ACCCTTGCTT GGTGCTGAG GTTGAGGGTG TTGTGGCTGG TATTGCTTAC
9151 GCTGGGCCCT GGAAGGCTAG GAACGCTTAC GATTGGACAG TTGAGAGTAC
9201 TGTTTACGTG TCACATAGGC ATCAAAGGTT GGGCCTAGGA TCCACATTGT
9251 ACACACATTT GCTTAAGTCT ATGGAGGCGC AAGGTTTTAA GTCTGTGGTT
9301 GCTGTTATAG GCCTTCCAAA CGATCCATCT GTTAGGTTGC ATGAGGCTTT
9351 GGGATACACA GCCCGGGGTA CATTGCGCGC AGCTGGATAC AAGCATGGTG
9401 GATGGCATGA TGTTGGTTTT TGGCAAAGGG ATTTTGAGTT GCCAGCTCCT
9451 CCAAGGCCAG TTAGGCCAGT TACCCAGATC TGAGTCGACC TGCAGGCATG
9501 CCCGCTGAAA TCACCAGTCT CTCTCTACAA ATCTATCTCT CTCTATAATA
9551 ATGTGTGAGT AGTTCCCAGA TAAGGGAATT AGGGTTCTTA TAGGGTTTCC
9601 CTCATGTGTT GAGCATATAA GAAACCCTTA GTATGTATTT GTATTGTAA
9651 AATACTCTA TCAATAAAAT TTCTAATTCC TAAAACCAAA ATCCAGGGCG
9701 AGCTCGGTAC CCGGGGATCC TCTAGAGTCG ACCTGCAGGC ATGCCCGCGG
9751 ATATCGATGG GCCCCGGCCG AAGCTTCGGT CCGGGCCATC GTGGCCTCTT
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U.S. Patent

Jun. 7, 2011

Sheet 5 of 7

US 7,956,246 B2

FIG. 1 (Sheet 5 of 5)

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9801 GCTCTTCAGG ATGAAGAGCT ATGTTTAAAC GTGCAAGCGC TCAATTTCGCC
9851 CTATAGTGAG TCGTATTACA ATCGTACGCA ATTCACTACA TTAAAAACGT
9901 CCGCAATGTG TTATTAAGTT GTCTAAGCGT CAATTTTTCC CTTCTATGGT
9951 CCCGTTTGTT TATCCTCTAA ATTATATAAT CCAGCTTAAA TAAGTTAAGA
10001 GACAAACAAA CAACACAGAT TATTAAATAG ATTATGTAAT CTAGATACCT
10051 AGATTATGTA ATCCATAAGT AGAATATCAG GTGCTTATAT AATCTATGAG
10101 CTCGATTATA TAATCTTAAA AGAAAACAAA CAGAGCCCCC ATAAAAAGGG
10151 GTCAAGTGGG CACTTGCTCA CTCATTAAAT CCCTCCCTCT CCTCTTTTAT
10201 CCCTCTTTTT GGTGTATTCA CCAATAGTGG TGTGCACCTG TGATTGGCTC
10251 GTAAAAATTC TTGGACGGAT GGAAGAGTGA AGAGATAAGC AAGTCAAAGA
10301 AAAGTAACAA CGAAGCTTCA TCAGCTACAA ATTTTGGCCC AACTGGTTCG
10351 ACCAGCACCA AACTTACGTA TACATGATTA TCTCTGTTTC CCTCATTTCG
10401 AAGAAAAAAA CGGGTTTCAA AACCCACTGC TTTCAGGAGT AAAAAAGAT
10451 AATAATCTGA AACATTGCTT CCACCTTGGC CCTTATTTCG TTACGTTGCA
10501 ATTCACCCCA ATCCACATGT GGATTGAGAT GGATTGCACT GTAGCTAGAC
10551 AAACCCTTAG GCCCTGTTTG CATAGGAATA CACCAGGAAT TATTCAGCT
10601 AATCAAAATT TATATAAATG AGAGAAACAA TTCGGATAGG AATGTTCCTA
10651 GGACTTCATT CTGCAGTAAC CGAACGGCCC CTTAATCCAC CCCAATACAC
10701 GTGGATTGGA GTGGATTGAG GTACAGCCAA ACAAGGCCA AGTGCAGATC
10751 AAATAAATCA CCCGTCATAT TCTTCTACCT ACAAAAACAG CAATAAACAC
10801 CTGAATGAAG TTCTAATTTG CACAGTGTAG GTAGGATGAA AATAGTTACC
10851 TCCTCATGGT CAGTAACCTT TGGCACACAA CTTACATGT AATCGATGTA
10901 CCACCTGGCT CTTGCCCTGAA ACCCAATACA TCTTTAGCAT AAGAATAATA
10951 TTATGATGGC AAGGCATGAT CACCAGCACT CCTTTATTGT TTAGTAAGTC
11001 TATCACTCCC CAAAACAATT CAAATGAACA GAGATGCATT GCCCCCAATG
11051 AATTCTATTT CAATTAGCCG GAAAAATCTA CTTCAATCAGA AGCATCCAAA
11101 TTGCCAGCAT CCCTACTAGA CTGACCATGA CCAGGCTGCC GCAGATGCCT
11151 CTTTTCTGT OCTCTCCTCT TTGCCCTGAG TTTCTCTTCA AGATCCCTCA
11201 CCCACGCTCT CTTATACATC TTAAAGCTAA CATGTCTCTC CTCGGCCATC
11251 TTCTTAACCT TCTCAGTAAT CTCAGCAGCA ATCTGACGGT TGTACAACTT
11301 CTTCAGCCCC TTCATCAACT TTGCAAAATG GTGAGGCTGT GGCATCAGTC
11351 CTGCCTCTAG CATGTCTAAG CAATACAGGC AGGCCTCCTT GACATGTTTC
11401 TTCGCAAAAC GTGCATGAAT CCAGATAGTC CATGCACTCA CATFGAGCTC
11451 ACAGCCTTTG CTCACAATAC ATTTCCAAAC ATCTTTTGA AGCTCAAGTT
11501 TCTCATCTCT GACCAACGCA TTGAGGAGGT CCTTCAGCAC CCCATATTGC
11551 GGTACCACAA AGAGCCCCCT CCCAACCATG TCTTTAAAT AACTACATGC
11601 CTCAATCAGC AAACCTGCCC CAACAAGGCC ACTCACCACG ATAGCAAATG
11651 TATCGACCAC AGGACTGAGC CCAGCACTTT CCATCTCATT CCACAATGTC
11701 ATGGCTTGCT TGGTCTCCCC AAGCCTGCAG GCCAACCAGG TCACCACATT
11751 GTATATCTTG AGATCTGGTG GACACCGGCA CTCCCGCATC CTCTCCATCA
11801 GCTCCAAGCA CTCCTCAAGC TGCTCCTTCT TCTCGTGTGC TACAAAGAAA
11851 CCATGGTACA CGGCAGCGTC CACCCGAGG CCATCCCTCG ACATAGCATC
11901 CAAGAACCTG TACCCCTGGG AT

```

Figure 2

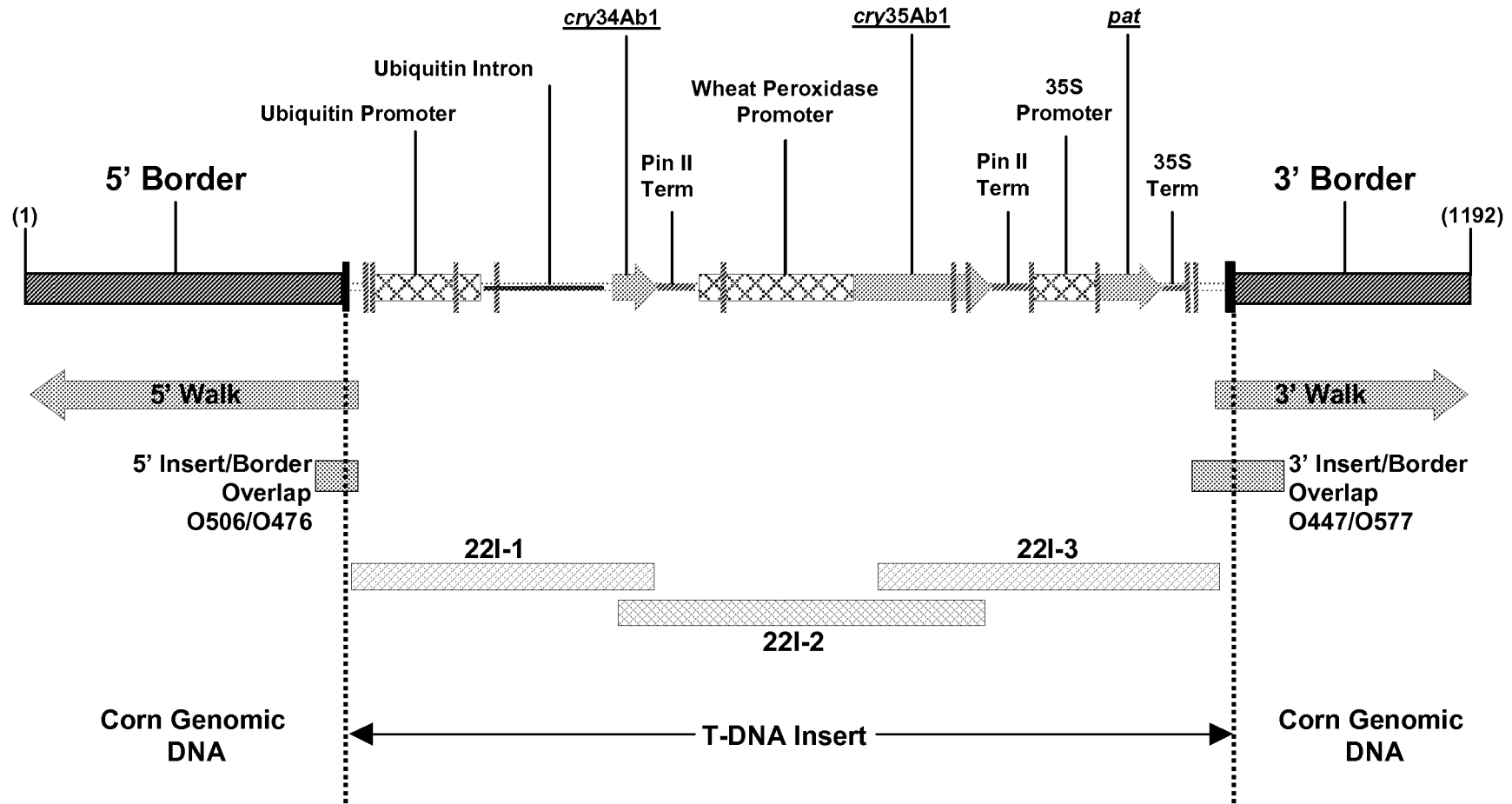
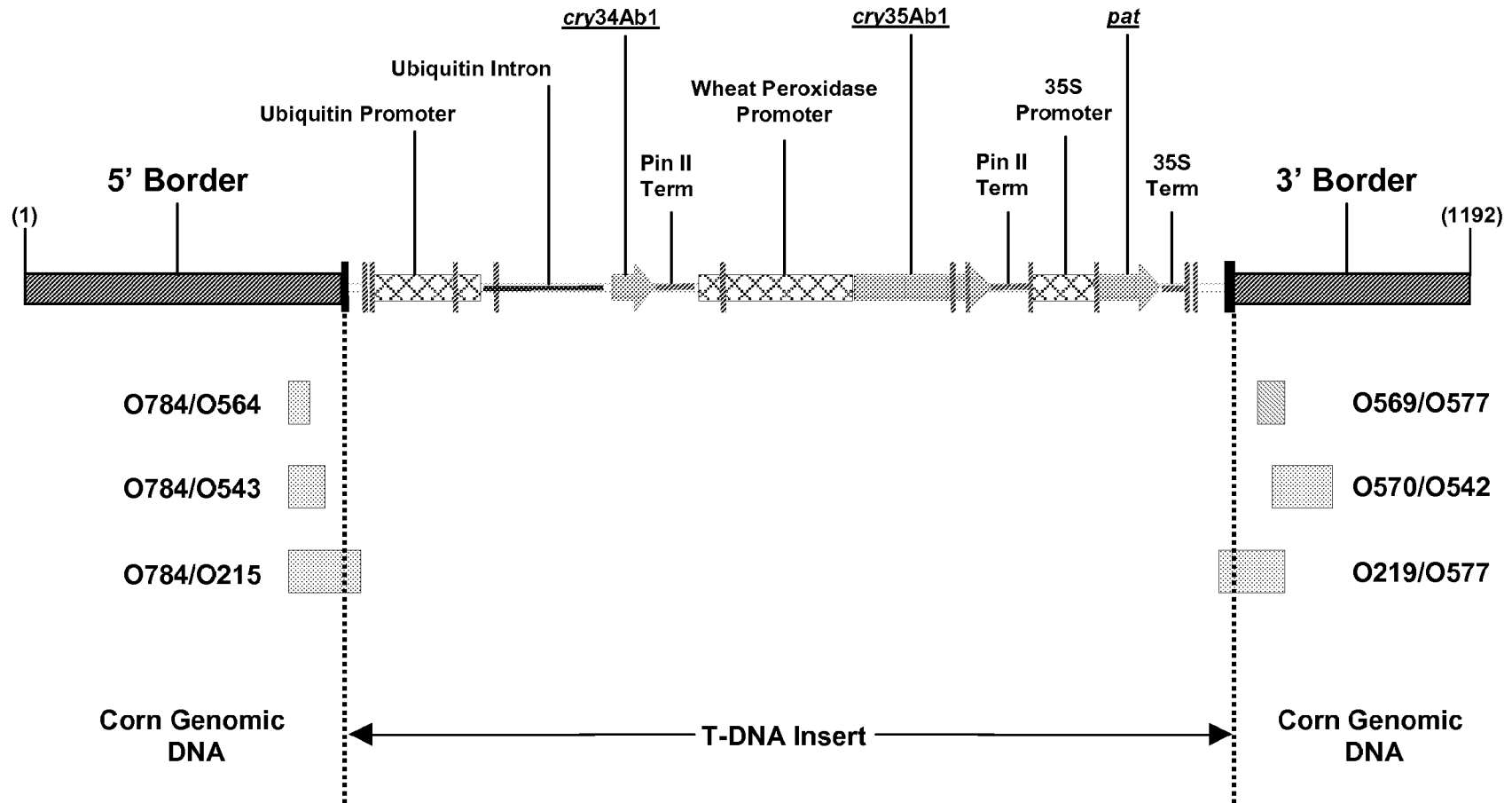




Figure 3



US 7,956,246 B2

1

# **CORN EVENT DAS-59122-7 AND METHODS FOR DETECTION THEREOF**

## **CROSS-REFERENCE TO RELATED APPLICATIONS**

This application is a divisional of U.S. Ser. No. 11/237,222 filed Sep. 28, 2005, now U.S. Pat. No. 7,323,556, which claims the benefit of U.S. Provisional Application Ser. No. 60/614,225, filed Sep. 29, 2004, the contents of which are herein incorporated by reference in their entirety.

## **FIELD OF INVENTION**

Embodiments of the present invention relate to the field of plant molecular biology, specifically an embodiment of the invention relates to a DNA construct for conferring insect resistance to a plant. Embodiments of the invention more specifically relate to an insect resistant corn plant DAS-59122-7 and to assays for detecting the presence of corn plant DAS-59122-7 DNA in a sample and compositions thereof.

## **BACKGROUND OF INVENTION**

An embodiment of this invention relates to the insect resistant corn (*Zea mays*) plant DAS-59122-7, also referred to as maize line DAS-59122-7 or maize event DAS-59122-7, and to the DNA plant expression construct of corn plant DAS-59122-7 and the detection of the transgene/flanking insertion region in corn plant DAS-59122-7 and progeny thereof.

Corn is an important crop and is a primary food source in many areas of the world. Damage caused by insect pests is a major factor in the loss of the world's corn crops, despite the use of protective measures such as chemical pesticides. In view of this, insect resistance has been genetically engineered into crops such as corn in order to control insect damage and to reduce the need for traditional chemical pesticides. One group of genes which have been utilized for the production of transgenic insect resistant crops are the delta-endotoxins from *Bacillus thuringiensis* (B.t.). Delta-endotoxins have been successfully expressed in crop plants such as cotton, potatoes, rice, sunflower, as well as corn, and have proven to provide excellent control over insect pests. (Perlak, F. J et al (1990) *Bio/Technology* 8, 939-943; Perlak, F. J. et al. (1993) *Plant Mol. Biol.* 22: 313-321; Fujimoto H. et al. (1993) *Bio/Technology* 11: 1151-1155; Tu et al. (2000) *Nature Biotechnology* 18:1101-1104; PCT publication number WO 01/13731; and Bing J W et al. (2000) Efficacy of Cry1F Transgenic Maize, 14<sup>th</sup> Biennial International Plant Resistance to Insects Workshop, Fort Collins, Colo.).

The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site (Weising et al., *Ann. Rev. Genet* 22:421-477, 1988). At the same time the presence of the transgene at different locations in the genome will influence the overall phenotype of the plant in different ways. For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of an introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant tissues, that may not correspond to the patterns expected from

2

transcriptional regulatory elements present in the introduced gene construct. For this reason, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual outcrossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure reliable gene expression in a number of varieties that are well adapted to local growing conditions.

It would be advantageous to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene of interest. In addition, a method for detecting a particular event would be helpful for complying with regulations requiring the pre-market approval and labeling of foods derived from recombinant crop plants, for example, or for use in environmental monitoring, monitoring traits in crops in the field, or monitoring products derived from a crop harvest, as well as for use in ensuring compliance of parties subject to regulatory or contractual terms.

It is possible to detect the presence of a transgene by any nucleic acid detection method known in the art including, but not limited to, the polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc., because for many DNA constructs, the coding region is interchangeable. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known. For example, an event-specific PCR assay is described in U.S. Pat. No. 6,395,485 for the detection of elite event GAT-ZM1. Accordingly, it would be desirable to have a simple and discriminative method for the identification of event DAS-59122-7.

## **SUMMARY OF INVENTION**

Embodiments of this invention relate to methods for producing and selecting an insect resistant monocot crop plant. More specifically, a DNA construct is provided that when expressed in plant cells and plants confers resistance to insects. According to one aspect of the invention, a DNA construct, capable of introduction into and replication in a host cell, is provided that when expressed in plant cells and plants confers insect resistance to the plant cells and plants. The DNA construct is comprised of a DNA molecule named PH117662A and it includes three (3) transgene expression cassettes. The first expression cassette comprises a DNA molecule which includes the promoter, 5' untranslated exon, and first intron of the maize ubiquitin (Ubi-1) gene (Christensen et al. (1992) *Plant Mol. Biol.* 18:675-689 and Christensen and Quail (1996) *Transgenic Res.* 5:213-218) operably connected to a DNA molecule encoding a B.t.  $\delta$ -endotoxin identified as Cry34Ab1 (U.S. Pat. Nos. 6,127,180, 6,624,145 and 6,340,593) operably connected to a DNA molecule comprising a Pin II transcriptional terminator isolated from potato (Gyheung An et al. (1989) *Plant Cell.* 1:115-122). The second transgene expression cassette of the DNA construct comprises a DNA molecule encoding the wheat peroxidase promoter (Hertig et al. (1991) *Plant Mol. Biol.* 16:171-174) operably connected to a DNA molecule encoding a B.t.  $\delta$ -endotoxin identified as Cry35Ab1 (U.S. Pat. Nos. 6,083,499,

## US 7,956,246 B2

3

6,548,291 and 6,340,593) operably connected to a DNA molecule comprising a Pin II transcriptional terminator isolated from potato (Gyheung An et al. (1989) *Plant Cell*. 1:115-122). The third transgene expression cassette of the DNA construct comprises a DNA molecule of the cauliflower mosaic virus (CaMV) 35S promoter (Odell J. T. et al. (1985) *Nature* 313: 810-812; Mitsuhara et al. (1996) *Plant Cell Physiol.* 37: 49-59) operably connected to a DNA molecule encoding a phosphinothricin acetyltransferase (PAT) gene (Wohleben W. et al. (1988) *Gene* 70: 25-37) operably connected to a DNA molecule comprising a 3' transcriptional terminator from (CaMV) 35S (see Mitsuhara et al. (1996) *Plant Cell Physiol.* 37: 49-59). Plants containing the DNA construct are also provided.

According to another embodiment of the invention, compositions and methods are provided for identifying a novel corn plant designated DAS-59122-7, which methods are based on primers or probes which specifically recognize the 5' and/or 3' flanking sequence of DAS-59122-7. DNA molecules are provided that comprise primer sequences that when utilized in a PCR reaction will produce amplicons unique to the transgenic event DAS-59122-7. These molecules may be selected from the group consisting of:

5'-GTGGCTCCTTCAACGTTGCGGTTCTGTGTC-3' (SEQ ID NO: 1);  
 5'-CGTGCAAGCGCTCAATTCGCCCTATAGTG-3' (SEQ ID NO: 2);  
 5'-AATTGAGCGCTTGACGTTT-3' (SEQ ID NO: 3);  
 5'-ACAACAAGACCGGCCACACCCTC-3' (SEQ ID NO: 4);  
 5'-GAGGTGGTCTGGATGGTGTAGGTCA-3' (SEQ ID NO: 5);  
 5'-TACAACCTCAAGTGGTTCCTCTTCCCGA-3' (SEQ ID NO: 6);  
 5'-GAGGTCTGGATCTGCATGATGCGGA-3' (SEQ ID NO: 7);  
 5'-AACCTTAGTATGTATTTGTATT-3' (SEQ ID NO: 8);  
 5'-CTCCTTCAACGTTGCGGTTCTGTGTCAG-3' (SEQ ID NO: 9);  
 5'-TTTTGCAAAGCGAAGATTGAGATG-3' (SEQ ID NO: 10);  
 5'-GCGGGACAAGCCGTTTACGTTT-3' (SEQ ID NO: 11);  
 5'-GACGGGTGATTTATTTGATCTGCAC-3' (SEQ ID NO: 12);  
 5'-CATCTGAATCGTTCGCTTTGCAAAA-3' (SEQ ID NO: 13);  
 5'-CTACGTTCCAATGGAGCTCGACTGTC-3' (SEQ ID NO: 14);  
 5'-GGTCAAGTGGACACTTGGTCACTCA-3' (SEQ ID NO: 15);  
 5'-GAGTGAAGAGATAAGCAAGTCAAAG-3' (SEQ ID NO: 16);  
 5'-CATGTATACGTAAGTTTGGTGTCTGG-3' (SEQ ID NO: 17);  
 5'-AATCCACAAGATTGGAGCAAACGAC-3' (SEQ ID NO: 18);  
 5'-CGTATTACAATCGTACGCAATTCAG-3' (SEQ ID NO: 36);  
 5'-GGATAAACAACGGGACCATAGAAG-3' (SEQ ID NO: 37) and complements thereof. The corn plant and seed comprising these molecules is an embodiment of this invention. Further, kits utilizing these primer sequences for the identification of the DAS-59122-7 event are provided.

An additional embodiment of the invention relates to the specific flanking sequences of DAS-59122-7 described herein, which can be used to develop specific identification

4

methods for DAS-59122-7 in biological samples. More particularly, the invention relates to the 5' and/or 3' flanking regions of DAS-59122-7, SEQ ID NO: 19, 5' flanking and SEQ ID NO: 20, 3' flanking, respectively, which can be used for the development of specific primers and probes. A further embodiment of the invention relates to identification methods for the presence of DAS-59122-7 in biological samples based on the use of such specific primers or probes.

According to another embodiment of the invention, methods of detecting the presence of DNA corresponding to the corn event DAS-59122-7 in a sample are provided. Such methods comprise: (a) contacting the sample comprising DNA with a DNA primer set, that when used in a nucleic acid amplification reaction with genomic DNA extracted from corn event DAS-59122-7 produces an amplicon that is diagnostic for corn event DAS-59122-7; (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon.

DNA molecules that comprise the novel transgene/flanking insertion region, SEQ ID NO: 21, 5' flanking plus 1000 internal and SEQ ID NO: 22, 3' flanking plus 1000 internal and are homologous or complementary to SEQ ID NO: 21 and SEQ ID NO: 22 are an embodiment of this invention.

DNA sequences that comprise the novel transgene/flanking insertion region, SEQ ID NO: 21 are an embodiment of this invention. DNA sequences that comprise a sufficient length of polynucleotides of transgene insert sequence and a sufficient length of polynucleotides of maize genomic and/or flanking sequence from maize plant DAS-59122-7 of SEQ ID NO: 21 that are useful as primer sequences for the production of an amplicon product diagnostic for maize plant DAS-59122-7 are included.

In addition, DNA sequences that comprise the novel transgene/flanking insertion region, SEQ ID NO: 22 are provided. DNA sequences that comprise a sufficient length of polynucleotides of transgene insert sequence and a sufficient length of polynucleotides of maize genomic and/or flanking sequence from maize plant DAS-59122-7 of SEQ ID NO: 22 that are useful as primer sequences for the production of an amplicon product diagnostic for maize plant DAS-59122-7 are included.

According to another embodiment of the invention, the DNA sequences that comprise at least 11 or more nucleotides of the transgene portion of the DNA sequence of SEQ ID NO: 21 or complements thereof, and a similar length of 5' flanking maize DNA sequence of SEQ ID NO: 21 or complements thereof are useful as DNA primers in DNA amplification methods. The amplicons produced using these primers are diagnostic for maize event DAS-59122-7. Therefore, embodiments of the invention also include the amplicons produced by DNA primers homologous or complementary to SEQ ID NO: 21.

According to another embodiment of the invention, the DNA sequences that comprise at least 11 or more nucleotides of the transgene portion of the DNA sequence of SEQ ID NO: 22 or complements thereof, and a similar length of 3' flanking maize DNA sequence of SEQ ID NO: 22 or complements thereof are useful as DNA primers in DNA amplification methods. The amplicons produced using these primers are diagnostic for maize event DAS-59122-7. Therefore, embodiments of the invention also include the amplicons produced by DNA primers homologous or complementary to SEQ ID NO: 22.

More specifically, a pair of DNA molecules comprising a DNA primer set, wherein the DNA molecules are identified as SEQ ID NO: 18 or complements thereof and SEQ ID NO: 1 or complements thereof; SEQ ID NO: 2 or complements

## US 7,956,246 B2

5

thereof and SEQ ID NO: 17 or complements thereof; SEQ ID NO: 10 or complements thereof and SEQ ID NO: 9 or complements thereof; SEQ ID NO: 8 or complements thereof and SEQ ID NO: 17 or complements thereof; and SEQ ID NO: 36 or complements thereof and SEQ ID NO: 37 or complements thereof are embodiments of the invention.

Further embodiments of the invention include the amplicon comprising the DNA molecules of SEQ ID NO: 18 and SEQ ID NO: 1; the amplicon comprising the DNA molecules of SEQ ID NO: 2 and SEQ ID NO: 17; the amplicon comprising the DNA molecules of SEQ ID NO: 10 and SEQ ID NO: 9; the amplicon comprising the DNA molecules of SEQ ID NO: 8 and SEQ ID NO: 17; and the amplicon comprising the DNA molecules of SEQ ID NO: 36 and SEQ ID NO: 37.

Further embodiments of the invention include the following primers, which are useful in detecting or characterizing event DAS-59122-7: SEQ ID NO: 11 or complements thereof; SEQ ID NO: 5 or complements thereof; SEQ ID NO: 4 or complements thereof; SEQ ID NO: 7 or complements thereof; SEQ ID NO: 6 or complements thereof; SEQ ID NO: 3 or complements thereof; SEQ ID NO: 18 or complements thereof; SEQ ID NO: 14 or complements thereof; SEQ ID NO: 13 or complements thereof; SEQ ID NO: 15 or complements thereof; SEQ ID NO: 17 or complements thereof; SEQ ID NO: 16 or complements thereof; and SEQ ID NO: 12 or complements thereof. Further embodiments also include the amplicons produced by pairing any of the primers listed above.

According to another embodiment of the invention, methods of detecting the presence of a DNA molecule corresponding to the DAS-59122-7 event in a sample, such methods comprising: (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe, molecule that hybridizes under stringent hybridization conditions with DNA extracted from corn event DAS-59122-7 and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA. More specifically, a method for detecting the presence of a DNA molecule corresponding to the DAS-59122-7 event in a sample, such methods, consisting of (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that consists of sequences that are unique to the event, e.g. junction sequences, wherein said DNA probe molecule hybridizes under stringent hybridization conditions with DNA extracted from corn event DAS-59122-7 and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.

In addition, a kit and methods for identifying event DAS-59122-7 in a biological sample which detects a DAS-59122-7 specific region within SEQ ID NO: 23 are provided.

DNA molecules are provided that comprise at least one junction sequence of DAS-59122-7 selected from the group consisting of SEQ ID NO: 32, 33, 34, and 35 and complements thereof; wherein a junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the corn cell flanking the insertion site, i.e. flanking DNA, and is diagnostic for the DAS-59122-7 event.

According to another embodiment of the invention, methods of producing an insect resistant corn plant that comprise the steps of: (a) sexually crossing a first parental corn line comprising the expression cassettes of the invention, which confers resistance to insects, and a second parental corn line that lacks insect resistance, thereby producing a plurality of

6

progeny plants; and (b) selecting a progeny plant that is insect resistant. Such methods may optionally comprise the further step of back-crossing the progeny plant to the second parental corn line to producing a true-breeding corn plant that is insect resistant.

A further embodiment of the invention provides a method of producing a corn plant that is resistant to insects comprising transforming a corn cell with the DNA construct PH117662A (SEQ ID NO: 24), growing the transformed corn cell into a corn plant, selecting the corn plant that shows resistance to insects, and further growing the corn plant into a fertile corn plant. The fertile corn plant can be self pollinated or crossed with compatible corn varieties to produce insect resistant progeny.

Another embodiment of the invention further relates to a DNA detection kit for identifying maize event DAS-59122-7 in biological samples. The kit comprises a first primer which specifically recognizes the 5' or 3' flanking region of DAS-59122-7, and a second primer which specifically recognizes a sequence within the foreign DNA of DAS-59122-7, or within the flanking DNA, for use in a PCR identification protocol. A further embodiment of the invention relates to a kit for identifying event DAS-59122-7 in biological samples, which kit comprises a specific probe having a sequence which corresponds or is complementary to, a sequence having between 80% and 100% sequence identity with a specific region of event DAS-59122-7. The sequence of the probe corresponds to a specific region comprising part of the 5' or 3' flanking region of event DAS-59122-7.

The methods and kits encompassed by the embodiments of the present invention can be used for different purposes such as, but not limited to the following: to identify event DAS-59122-7 in plants, plant material or in products such as, but not limited to, food or feed products (fresh or processed) comprising, or derived from plant material; additionally or alternatively, the methods and kits can be used to identify transgenic plant material for purposes of segregation between transgenic and non-transgenic material; additionally or alternatively, the methods and kits can be used to determine the quality of plant material comprising maize event DAS-59122-7. The kits may also contain the reagents and materials necessary for the performance of the detection method.

A further embodiment of this invention relates to the DAS-59122-7 corn plant or its parts, including, but not limited to, pollen, ovules, vegetative cells, the nuclei of pollen cells, and the nuclei of egg cells of the corn plant DAS-59122-7 and the progeny derived thereof. The corn plant and seed DAS-59122-7 from which the DNA primer molecules provide a specific amplicon product is an embodiment of the invention.

The foregoing and other aspects of the invention will become more apparent from the following detailed description and accompanying drawing.

## BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. DNA sequence (SEQ ID NO: 23) showing the transgenic insert PH117662A, as well as the sequences flanking the transgenic insert. The 5' and 3' border regions, bp 1 to bp2593 and bp 9937 to bp 11922, respectively, are underlined. Two nucleotide differences (bp 6526 and bp 6562) based on comparison to the transforming plasmid PHP17662 are noted in bold and underlined.

FIG. 2. Schematic diagram of the B.t. Cry34/35Ab1 event DAS-59122-7 insert region is divided into three separate sections; the 5' border region with corn genomic DNA, the intact T-DNA insert, and the 3' border region with corn genomic DNA. The two arrows beneath the diagram of the



US 7,956,246 B2

7

insert indicate the start and end points of the sequence derived from 5' and 3' genome walking fragments. Other boxes beneath the diagram of the insert represent PCR fragments that were amplified from genomic DNA of event DAS-59122-7 and sequenced to cover the intact T-DNA insert and the 5' and 3' insert/border junction regions.

FIG. 3. Schematic diagram of the B.t. Cry34/35Ab1 event DAS-59122-7 insert region is divided into three separate sections; the 5' border region with corn genomic DNA, the intact T-DNA insert, and the 3' border region with corn genomic DNA. Boxes beneath the diagram of the insert represent PCR fragments located in either the genomic border regions or across the 5' and 3' junction regions of the T-DNA insert with corn genomic DNA that were amplified from genomic DNA from event DAS-59122-7.

#### DETAILED DESCRIPTION

The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger et al., *Glossary of Genetics: Classical and Molecular*, 5<sup>th</sup> edition, Springer-Verlag; New York, 1991; and Lewin, *Genes V*, Oxford University Press: New York, 1994. The nomenclature for DNA bases as set forth at 37 CFR §1.822 is used.

As used herein, the term “comprising” means “including but not limited to”.

As used herein, the term “corn” means *Zea mays* or maize and includes all plant varieties that can be bred with corn, including wild maize species.

As used herein, the term “DAS-59122-7 specific” refers to a nucleotide sequence which is suitable for discriminatively identifying event DAS-59122-7 in plants, plant material, or in products such as, but not limited to, food or feed products (fresh or processed) comprising, or derived from plant material.

As used herein, the terms “insect resistant” and “impacting insect pests” refers to effecting changes in insect feeding, growth, and/or behavior at any stage of development, including but not limited to: killing the insect; retarding growth; preventing reproductive capability; inhibiting feeding; and the like.

As used herein, the terms “pesticidal activity” and “insecticidal activity” are used synonymously to refer to activity of an organism or a substance (such as, for example, a protein) that can be measured by numerous parameters including, but not limited to, pest mortality, pest weight loss, pest attraction, pest repellency, and other behavioral and physical changes of a pest after feeding on and/or exposure to the organism or substance for an appropriate length of time. For example “pesticidal proteins” are proteins that display pesticidal activity by themselves or in combination with other proteins.

“Coding sequence” refers to a nucleotide sequence that codes for a specific amino acid sequence. As used herein, the terms “encoding” or “encoded” when used in the context of a specified nucleic acid mean that the nucleic acid comprises the requisite information to guide translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid or may lack such intervening non-translated sequences (e.g., as in cDNA).

8

“Gene” refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. “Native gene” refers to a gene as found in nature with its own regulatory sequences. “Chimeric gene” refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. “Endogenous gene” refers to a native gene in its natural location in the genome of an organism. “Foreign” refers to material not normally found in the location of interest. Thus “foreign DNA” may comprise both recombinant DNA as well as newly introduced, rearranged DNA of the plant. A “foreign” gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A “transgene” is a gene that has been introduced into the genome by a transformation procedure. The site in the plant genome where a recombinant DNA has been inserted may be referred to as the “insertion site” or “target site”.

As used herein, “insert DNA” refers to the heterologous DNA within the expression cassettes used to transform the plant material while “flanking DNA” can exist of either genomic DNA naturally present in an organism such as a plant, or foreign (heterologous) DNA introduced via the transformation process which is extraneous to the original insert DNA molecule, e.g. fragments associated with the transformation event. A “flanking region” or “flanking sequence” as used herein refers to a sequence of at least twenty (20) base pair, preferably at least fifty (50) base pair, and up to five thousand (5000) base pair which is located either immediately upstream of and contiguous with or immediately downstream of and contiguous with the original foreign insert DNA molecule. Transformation procedures leading to random integration of the foreign DNA will result in transformants containing different flanking regions characteristic and unique for each transformant. When recombinant DNA is introduced into a plant through traditional crossing, its flanking regions will generally not be changed. Transformants will also contain unique junctions between a piece of heterologous insert DNA and genomic DNA, or two (2) pieces of genomic DNA, or two (2) pieces of heterologous DNA. A “junction” is a point where two (2) specific DNA fragments join. For example, a junction exists where insert DNA joins flanking DNA. A junction point also exists in a transformed organism where two (2) DNA fragments join together in a manner that is modified from that found in the native organism. “Junction DNA” refers to DNA that comprises a junction point.

As used herein, “heterologous” in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous nucleotide sequence can be from a species different from that from which the nucleotide sequence was derived, or, if from the same species, the promoter is not naturally found operably linked to the nucleotide sequence. A heterologous protein may originate from a foreign species, or, if from the same species, is substantially modified from its original form by deliberate human intervention.

## US 7,956,246 B2

9

“Regulatory sequences” refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

“Promoter” refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements are often referred to as enhancers. Accordingly, an “enhancer” is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters that cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg (1989) *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, nucleic acid fragments of different lengths may have identical promoter activity.

The “translation leader sequence” refers to a nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect numerous parameters including, processing of the primary transcript to mRNA, mRNA stability and/or translation efficiency. Examples of translation leader sequences have been described (Turner and Foster (1995) *Mol. Biotechnol.* 3:225-236).

The “3' non-coding sequences” refer to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al. (1989) *Plant Cell* 1:671-680.

A “protein” or “polypeptide” is a chain of amino acids arranged in a specific order determined by the coding sequence in a polynucleotide encoding the polypeptide.

A DNA construct is an assembly of DNA molecules linked together that provide one or more expression cassettes. The DNA construct may be a plasmid that is enabled for self replication in a bacterial cell and contains various endonuclease enzyme restriction sites that are useful for introducing DNA molecules that provide functional genetic elements, i.e., promoters, introns, leaders, coding sequences, 3' termination regions, among others; or a DNA construct may be a linear assembly of DNA molecules, such as an expression cassette. The expression cassette contained within a DNA construct comprise the necessary genetic elements to provide transcrip-

10

tion of a messenger RNA. The expression cassette can be designed to express in prokaryote cells or eukaryotic cells. Expression cassettes of the embodiments of the present invention are designed to express in plant cells.

The DNA molecules of embodiments of the invention are provided in expression cassettes for expression in an organism of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a coding sequence. “Operably linked” means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. Operably linked is intended to indicate a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes or multiple DNA constructs.

The expression cassette will include in the 5' to 3' direction of transcription: a transcriptional and translational initiation region, a coding region, and a transcriptional and translational termination region functional in the organism serving as a host. The transcriptional initiation region (i.e., the promoter) may be native or analogous, or foreign or heterologous to the host organism. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation.

It is to be understood that as used herein the term “transgenic” includes any cell, cell line, callus, tissue, plant part, or plant, the genotype of which has been altered by the presence of a heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term “transgenic” as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

A transgenic “event” is produced by transformation of plant cells with a heterologous DNA construct(s), including a nucleic acid expression cassette that comprises a transgene of interest, the regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. An event is characterized phenotypically by the expression of the transgene. At the genetic level, an event is part of the genetic makeup of a plant. The term “event” also refers to progeny produced by a sexual outcross between the transformant and another variety that include the heterologous DNA. Even after repeated back-crossing to a recurrent parent, the inserted DNA and flanking DNA from the transformed parent is present in the progeny of the cross at the same chromosomal location. The term “event” also refers to DNA from the original transformant comprising the inserted DNA and flanking sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

## US 7,956,246 B2

11

An insect resistant DAS-59122-7 corn plant can be bred by first sexually crossing a first parental corn plant consisting of a corn plant grown from the transgenic DAS-59122-7 corn plant and progeny thereof derived from transformation with the expression cassettes of the embodiments of the present invention that confers insect resistance, and a second parental corn plant that lacks insect resistance, thereby producing a plurality of first progeny plants; and then selecting a first progeny plant that is resistant to insects; and selling the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants an insect resistant plant. These steps can further include the back-crossing of the first insect resistant progeny plant or the second insect resistant progeny plant to the second parental corn plant or a third parental corn plant, thereby producing a corn plant that is resistant to insects.

As used herein, the term “plant” includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and progeny of same. Parts of transgenic plants understood to be within the scope of the invention comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, leaves, and roots originating in transgenic plants or their progeny previously transformed with a DNA molecule of the invention and therefore consisting at least in part of transgenic cells, are also an embodiment of the present invention.

As used herein, the term “plant cell” includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants that can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

“Transformation” refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as “transgenic” organisms. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol.* 143:277) and particle-accelerated or “gene gun” transformation technology (Klein et al. (1987) *Nature* (London) 327:70-73; U.S. Pat. No. 4,945,050, incorporated herein by reference). Additional transformation methods are disclosed below.

Thus, isolated polynucleotides of the invention can be incorporated into recombinant constructs, typically DNA constructs, which are capable of introduction into and replication in a host cell. Such a construct can be a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., (1985; Supp. 1987) *Cloning Vectors: A Laboratory Manual*, Weissbach and Weissbach (1989) *Methods for Plant Molecular Biology*, (Academic Press, New York); and Flevin et al., (1990) *Plant Molecular Biology Manual*, (Kluwer Academic Publishers). Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome

12

binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for different traits and crops can be found in one of several references, e.g., Fehr, in *Breeding Methods for Cultivar Development*, Wilcos J. ed., American Society of Agronomy, Madison Wis. (1987).

A “probe” is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. Such a probe is complementary to a strand of a target nucleic acid, in the case of the present invention, to a strand of isolated DNA from corn event DAS-59122-7 whether from a corn plant or from a sample that includes DNA from the event. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

“Primers” are isolated nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs of the invention refer to their use for amplification of a target nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods. “PCR” or “polymerase chain reaction” is a technique used for the amplification of specific DNA segments (see, U.S. Pat. Nos. 4,683,195 and 4,800,159; herein incorporated by reference).

Probes and primers are of sufficient nucleotide length to bind to the target DNA sequence specifically in the hybridization conditions or reaction conditions determined by the operator. This length may be of any length that is of sufficient length to be useful in a detection method of choice. Generally, eleven (11) nucleotides or more in length, eighteen (18) nucleotides or more, and twenty-two (22) nucleotides or more, are used. Such probes and primers hybridize specifically to a target sequence under high stringency hybridization conditions. Probes and primers according to embodiments of the present invention may have complete DNA sequence similarity of contiguous nucleotides with the target sequence, although probes differing from the target DNA sequence and that retain the ability to hybridize to target DNA sequences may be designed by conventional methods. Probes can be used as primers, but are generally designed to bind to the target DNA or RNA and are not used in an amplification process.

Specific primers can be used to amplify an integration fragment to produce an amplicon that can be used as a “specific probe” for identifying event DAS-59122-7 in biological samples. When the probe is hybridized with the nucleic acids of a biological sample under conditions which allow for the binding of the probe to the sample, this binding can be detected and thus allow for an indication of the presence of event DAS-59122-7 in the biological sample. Such identification of a bound probe has been described in the art. In an embodiment of the invention the specific probe is a sequence which, under optimized conditions, hybridizes specifically to



US 7,956,246 B2

13

a region within the 5' or 3' flanking region of the event and also comprises a part of the foreign DNA contiguous therewith. The specific probe may comprise a sequence of at least 80%, between 80 and 85%, between 85 and 90%, between 90 and 95%, and between 95 and 100% identical (or complementary) to a specific region of the event.

Methods for preparing and using probes and primers are described, for example, in *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed., vol. 1-3, ed. Sambrook et al, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989 (hereinafter, "Sambrook et al, 1989"); *Current Protocols in Molecular Biology*, ed. Ausubel et al, Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, "Ausubel et al., 1992"); and Innis et al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as the PCR primer analysis tool in Vector NTI version 6 (Informax Inc., Bethesda Md.); PrimerSelect (DNASTAR Inc., Madison, Wis.); and Primer (Version 0.5©, 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). Additionally, the sequence can be visually scanned and primers manually identified using guidelines known to one of skill in the art.

A "kit" as used herein refers to a set of reagents for the purpose of performing the method embodiments of the invention, more particularly, the identification of the event DAS-59122-7 in biological samples. The kit of the invention can be used, and its components can be specifically adjusted, for purposes of quality control (e.g. purity of seed lots), detection of event DAS-59122-7 in plant material, or material comprising or derived from plant material, such as but not limited to food or feed products. "Plant material" as used herein refers to material which is obtained or derived from a plant.

Primers and probes based on the flanking DNA and insert sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed sequences by conventional methods, e.g., by re-cloning and sequencing such sequences. The nucleic acid probes and primers of the present invention hybridize under stringent conditions to a target DNA sequence. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. Nucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure.

A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook et al., 1989, and by Haymes et al., In: *Nucleic Acid Hybridization, a Practical Approach*, IRL Press, Washington, D.C. (1985), departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capac-

14

ity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

In hybridization reactions, specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. The thermal melting point ( $T_m$ ) is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. For DNA-DNA hybrids, the  $T_m$  can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284:  $T_m = 81.5^\circ \text{C} + 16.6 (\log M) + 0.41 (\% \text{GC}) - 0.61 (\% \text{form}) - 500/L$ ; where M is the molarity of monovalent cations, % GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs.  $T_m$  is reduced by about  $1^\circ \text{C}$ . for each 1% of mismatching; thus,  $T_m$ , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with >90% identity are sought, the  $T_m$  can be decreased  $10^\circ \text{C}$ . Generally, stringent conditions are selected to be about  $5^\circ \text{C}$ . lower than the  $T_m$  for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or  $4^\circ \text{C}$ . lower than the  $T_m$ ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or  $10^\circ \text{C}$ . lower than the  $T_m$ ; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or  $20^\circ \text{C}$ . lower than the  $T_m$ .

Using the equation, hybridization and wash compositions, and desired  $T_m$ , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a  $T_m$  of less than  $45^\circ \text{C}$ . (aqueous solution) or  $32^\circ \text{C}$ . (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes*, Part 1, Chapter 2 (Elsevier, N.Y.); and Ausubel et al., eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.).

As used herein, a substantially homologous sequence is a nucleic acid molecule that will specifically hybridize to the complement of the nucleic acid molecule to which it is being compared under high stringency conditions. Appropriate stringency conditions which promote DNA hybridization, for example,  $6\times$  sodium chloride/sodium citrate (SSC) at about  $45^\circ \text{C}$ ., followed by a wash of  $2\times \text{SSC}$  at  $50^\circ \text{C}$ ., are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about  $30^\circ \text{C}$ . for short probes (e.g., 10 to 50 nucleotides) and at least about  $60^\circ \text{C}$ . for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of a destabilizing agent such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dode-



US 7,956,246 B2

15

cyl sulphate) at 37° C., and a wash in 1× to 2×SSC (20× SSC=3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55° C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.5× to 1×SSC at 55 to 60° C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37° C., and a wash in 0.1×SSC at 60 to 65° C. A nucleic acid of the invention may specifically hybridize to one or more of the nucleic acid molecules unique to the DAS-59122-7 event or complements thereof or fragments of either under moderately stringent conditions.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local homology algorithm of Smith et al. (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0); the ALIGN PLUS program (version 3.0, copyright 1997); and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 10 (available from Accelrys, 9685 Scranton Road, San Diego, Calif. 92121, USA). Alignments using these programs can be performed using the default parameters.

The CLUSTAL program is well described by Higgins and Sharp, *Gene* 73: 237-244 (1988); Higgins and Sharp, *CABIOS* 5: 151-153 (1989); Corpet, et al., *Nucleic Acids Research* 16: 10881-90 (1988); Huang, et al., *Computer Applications in the Biosciences* 8: 155-65 (1992), and Pearson, et al., *Methods in Molecular Biology* 24: 307-331 (1994). The ALIGN and the ALIGN PLUS programs are based on the algorithm of Myers and Miller (1988) *supra*. The BLAST programs of Altschul et al. (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) *supra*. The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). Alignment may also be performed manually by visual inspection.

To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al. (1997) *supra*. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).

16

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

Regarding the amplification of a target nucleic acid sequence (e.g., by PCR) using a particular amplification primer pair, "stringent conditions" are conditions that permit the primer pair to hybridize only to the target nucleic acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product, the amplicon, in a DNA thermal amplification reaction.

The term "specific for (a target sequence)" indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

As used herein, "amplified DNA" or "amplicon" refers to the product of nucleic acid amplification of a target nucleic acid sequence that is part of a nucleic acid template. For example, to determine whether a corn plant resulting from a sexual cross contains transgenic event genomic DNA from the corn plant of the invention, DNA extracted from the corn plant tissue sample may be subjected to a nucleic acid amplification method using a DNA primer pair that includes a first primer derived from flanking sequence adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA. Alternatively, the second primer may be derived from the flanking sequence. The amplicon is of a length and has a sequence that is also diagnostic for the event. The amplicon

US 7,956,246 B2

17

may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. Alternatively, primer pairs can be derived from flanking sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence of the PH117662A expression construct as well as the sequence flanking the transgenic insert, see FIG. 1 (SEQ ID NO: 23), approximately twelve (12) Kb in size. A member of a primer pair derived from the flanking sequence may be located a distance from the inserted DNA sequence, this distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. The use of the term "amplicon" specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

Nucleic acid amplification can be accomplished by any of the various nucleic acid amplification methods known in the art, including the polymerase chain reaction (PCR). A variety of amplification methods are known in the art and are described, inter alia, in U.S. Pat. Nos. 4,683,195 and 4,683,202 and in *PCR Protocols: A Guide to Methods and Applications*, ed. Innis et al., Academic press, San Diego, 1990. PCR amplification methods have been developed to amplify up to 22 Kb of genomic DNA and up to 42 Kb of bacteriophage DNA (Cheng et al., *Proc. Natl. Acad. Sci. USA* 91:5695-5699, 1994). These methods as well as other methods known in the art of DNA amplification may be used in the practice of the embodiments of the present invention. It is understood that a number of parameters in a specific PCR protocol may need to be adjusted to specific laboratory conditions and may be slightly modified and yet allow for the collection of similar results. These adjustments will be apparent to a person skilled in the art.

The amplicon produced by these methods may be detected by a plurality of techniques, including, but not limited to, Genetic Bit Analysis (Nikiforov, et al. *Nucleic Acid Res.* 22:4167-4175, 1994) where a DNA oligonucleotide is designed which overlaps both the adjacent flanking DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking sequence) a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Another detection method is the Pyrosequencing technique as described by Winge (*Innov. Pharma. Tech.* 00: 18-24, 2000). In this method an oligonucleotide is designed that overlaps the adjacent DNA and insert DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. dNTPs are added individually and the incorporation results in a light signal which is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Fluorescence Polarization as described by Chen et al., (*Genome Res.* 9:492-498, 1999) is also a method that can be used to detect an amplicon of the invention. Using this method an

18

oligonucleotide is designed which overlaps the flanking and inserted DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking DNA sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Taqman® (PE Applied Biosystems, Foster City, Calif.) is described as a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by the manufacturer. Briefly, a FRET oligonucleotide probe is designed which overlaps the flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

Molecular Beacons have been described for use in sequence detection as described in Tyangi et al. (*Nature Biotech.* 14:303-308, 1996). Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

A hybridization reaction using a probe specific to a sequence found within the amplicon is yet another method used to detect the amplicon produced by a PCR reaction.

Embodiments of the present invention are further defined in the following Examples. It should be understood that these Examples are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the embodiments of the invention to adapt it to various usages and conditions. Thus, various modifications of the embodiments of the invention, in addition to those shown and described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

Applicants made a deposit of at least 2500 seeds of hybrid corn (maize) seed comprising event DAS-59122-7 on Oct. 4, 2010 with the American Type Culture Collection (ATCC), Manassas, VA 20110 USA. The deposit was assigned ATCC Patent Deposit Designation PTA-11384. Access to this deposit will be available during the pendency of the application to the Commissioner of Patents and Trademarks and persons determined by the Commissioner to be entitled thereto upon request. Upon the grant of a patent from the present application, Applicants will make the deposit avail-

## US 7,956,246 B2

19

able to the public pursuant to 37 C.F.R. § 1.808. This deposit of hybrid corn (maize) seed comprising event DAS-59122-7 will be maintained in the ATCC depository, which is a public depository, for a period of 30 years, or 5 years after the most recent request, or for the enforceable life of the patent, whichever is longer, and will be replaced if it becomes nonviable during that period. Additionally, Applicant has or will satisfy all the requirements of 37 C.F.R. §§1.801 - 1.809, including providing an indication of the viability of the sample upon deposit. Applicant has no authority to waive any restrictions imposed by law on the transfer of biological material or its transportation in commerce. Applicant does not waive any infringement of their rights granted under this patent or under the Plant Variety Protection Act (7 U.S.C § 2321 et seq.).

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

## EXAMPLES

## Example 1

Transformation of Maize by *Agrobacterium*  
Transformation and Regeneration of Transgenic  
Plants Containing the Cry34Ab1 and Cry35Ab1  
(Cry34/35Ab1) Genes

A DNA molecule of approximately 7.4 Kb, designated PH117662A (SEQ ID NO: 24), which includes a first transgene expression cassette comprising a DNA molecule which includes the promoter, 5' untranslated exon, and first intron of the maize ubiquitin (Ubi-1) gene (Christensen et al. (1992) *Plant Mol. Biol.* 18:675-689 and Christensen and Quail (1996) *Transgenic Res.* 5:213-218) operably connected to a DNA molecule encoding a B.t.  $\delta$ -endotoxin identified as Cry34Ab1 (U.S. Pat. Nos. 6,127,180, 6,624,145 and 6,340,593) operably connected to a DNA molecule comprising a Pin II transcriptional terminator isolated from potato (Gyheung An et al. (1989) *Plant Cell.* 1:115-122). The second transgene expression cassette of the DNA construct comprises a DNA molecule encoding the wheat peroxidase promoter (Hertig et al. (1991) *Plant Mol. Biol.* 16:171-174) operably connected to a DNA molecule encoding a B.t.  $\delta$ -endotoxin identified as Cry35Ab1 (U.S. Pat. Nos. 6,083,499, 6,548,291 and 6,340,593) operably connected to a DNA molecule comprising a Pin II transcriptional terminator isolated from potato (Gyheung An et al. (1989) *Plant Cell.* 1:115-122). The third transgene expression cassette of the DNA construct comprises a DNA molecule of the cauliflower mosaic virus (CaMV) 35S promoter (Odell J. T. et al. (1985) *Nature* 313: 810-812; Mitsuhashi et al. (1996) *Plant Cell Physiol.* 37: 49-59) operably connected to a DNA molecule encoding a phosphinothricin acetyltransferase (PAT) gene (Wohlleben W. et al. (1988) *Gene* 70: 25-37) operably connected to a DNA molecule comprising a 3' transcriptional terminator from (CaMV) 35S (see Mitsuhashi et al. (1996) *Plant Cell Physiol.* 37: 49-59) was used to transform maize embryo tissue.

B.t. Cry34/35 Ab1 maize plants were obtained by *Agrobacterium* transformation, the method of Zhao was employed (U.S. Pat. No. 5,981,840, and PCT patent publication WO98/32326; the contents of which are hereby incorporated by reference). Briefly, immature embryos were isolated from maize and the embryos contacted with a suspension of *Agrobacterium*, where the bacteria was capable of transferring PH117662 DNA (SEQ ID NO:24) to at least one cell of at least one of the immature embryos (step 1: the infection step). Specifically, in this step the immature embryos were

20

immersed in an *Agrobacterium* suspension for the initiation of inoculation. The embryos were co-cultured for a time with the *Agrobacterium* (step 2: the co-cultivation step). Specifically, the immature embryos were cultured on solid medium following the infection step. Following this co-cultivation period a "resting" step was provided. In this resting step, the embryos were incubated in the presence of at least one antibiotic known to inhibit the growth of *Agrobacterium* without the addition of a selective agent for plant transformants (step 3: resting step). In particular, the immature embryos are cultured on solid medium with antibiotic, but without a selecting agent, for elimination of *Agrobacterium* and for a resting phase for the infected cells. Next, inoculated embryos were cultured on medium containing a selective agent and growing transformed callus was recovered (step 4: the selection step). Specifically, the immature embryos were cultured on solid medium with a selective agent resulting in the selective growth of transformed cells. The callus was then regenerated into plants (step 5: the regeneration step), and, specifically, calli grown on selective medium were cultured on solid medium to regenerate the plants. Individual embryos were kept physically separate during culture, and the majority of explants died on the selective medium.

Those embryos that survived and produced healthy, glufosinate-resistant callus tissue were assigned unique identification codes representing putative transformation events, and continually transferred to fresh selection medium. Plants were regenerated from tissue derived from each unique event and transferred to the greenhouse. Leaf samples were taken for molecular analysis to verify the presence of the transgene by PCR and to confirm expression of the Cry34/35Ab1 protein by ELISA. Plants were then subjected to a whole plant bioassay using western corn rootworm insects. Positive plants were crossed with inbred lines to obtain seed from the initial transformed plants. A number of lines were evaluated in the field. The DAS-59122-7 event was selected from a population of independent transgenic events based on a superior combination of characteristics, including insect resistance and agronomic performance.

## Example 2

Identification of *Bacillus thuringiensis* Cry34/35Ab1  
Maize Line DAS-59122-7

Seed from event DAS-59122-7 was evaluated. The T1S2 seed represents transformation into the Hi-11 background, followed by a cross with inbred line PH09B and two rounds of self-crossing. All seed were obtained from Pioneer Hi-Bred (Johnston, Iowa). Primary characterization was conducted on plant leaf tissue during the study by confirmation of phosphinothricin acetyltransferase (PAT) activity via herbicide leaf painting and Cry34Ab1 expression using lateral flow devices.

Control substances in this study were defined as unmodified seed representative of the test substance background. Control seeds of Hi-11 and PH09B backgrounds were used as negative controls. These unmodified seed do not contain the plant transcription units for the cry34Ab1, cry35Ab1, and pat genes. All seed were obtained from Pioneer Hi-Bred (Johnston, Iowa).

DNA samples from two additional B.t. Cry34/35Ab1 events, event DAS-45214-4 and event DAS-45216-6, were used as negative controls for event specific PCR analysis. The two events were produced through *Agrobacterium* transformation using the same vector used to produce event DAS-59122-7 and therefore contained the plant transcription units for the cry34Ab1, cry35Ab1, and pat genes. However, the



## US 7,956,246 B2

## 21

insertions sites of the T-DNA in events DAS-45214-4 and DAS-45216-6, including genomic DNA border regions, were different from that in event DAS-59122-7. DNA samples from event DAS-45214-4 and event DAS-45216-6 were isolated and characterized by Southern blot analysis. (Data not shown.)

Corn seed for event DAS-59122-7 and unmodified control seed (Hi-11 and PH09B) were planted in growth chambers at the DuPont Experimental Station (Wilmington, Del.) to produce sufficient numbers of plants for DNA analysis. For characterization of event DAS-59122-7, ten (10) T1S2 seeds were planted. Ten (10) seeds were also planted for each unmodified control line. One (1) seed was planted per pot, and the pot was uniquely identified. Planting and growing conditions were conducive to healthy plant growth including regulated light and water.

Leaf samples were collected for each of the control and event DAS-59122-7 plants. For each sample, sufficient leaf material from above the growing point was collected and placed in a pre-labeled sample bag. The samples were placed

## 22

to confirm the identity of the test substance plants prior to molecular analysis as shown in Table 1.

To confirm the expression of phosphinothricin acetyltransferase (PAT) in event DAS-59122-7 plants, herbicide leaf painting was conducted. All plants used in this study were leaf painted to confirm plant identity. Plants were assayed prior to the R1 growth stage. Assays were conducted following a standard procedure known in the art for herbicide leaf painting for the identification of PAT-expressing transgenic plants. Specifically, a portion of one leaf of each plant was treated with approximately 2% solution of glufosinate herbicide, Basta® (Bayer CropScience) in water and visually checked for brown or necrotic tissue in the painted leaf area 4-12 days after application. Results for each plant were recorded and used to determine expression of PAT in each test plant as shown in Table 1. As shown in Table 1, of the ten (10) plants tested for event DAS-59122-7 T1S2 generation, six (6) plants expressed both Cry34Ab1 and PAT, while four (4) plants did not express either protein. All unmodified controls tested negative for both CryAb1 And PAT assays (data not shown).

TABLE 1

Cry34Ab1 and PAT Protein Expression and Southern Hybridization Data for B.t. Cry34/35Ab1 Event DAS-59122-7					
Plant ID	Sample ID	Cry34Ab1 and PAT Expression <sup>1</sup>	Southern Blot cry34Ab1 Probe <sup>2</sup>	Southern Blot cry35Ab1 Probe <sup>2</sup>	Southern Blot pat Probe <sup>2</sup>
02-122C 1	DAS59122-7 T1S2 1	positive	+	+	+
02-122C 2	DAS59122-7 T1S2 2	positive	+	+	+
02-122C 3	DAS59122-7 T1S2 3	positive	+	+	+
02-122C 4	DAS59122-7 T1S2 4	negative	-	-	-
02-122C 5	DAS59122-7 T1S2 5	positive	+	+	+
02-122C 6	DAS59122-7 T1S2 6	negative	-	-	-
02-122C 7	DAS59122-7 T1S2 7	positive	+	+	+
02-122C 8	DAS59122-7 T1S2 8	negative	-	-	-
02-122C 9	DAS59122-7 T1S2 9	negative	-	-	-
02-122C 10	DAS59122-7 T1S2 10	positive	+	+	+

<sup>1</sup>Positive Cry34Ab1 expression indicates detection of protein expression as determined by the immunoassay-based lateral flow device specific for Cry34Ab1 protein detection. Negative indicates no detection of the Cry34Ab1 protein. Positive PAT expression indicates plants that were tolerant to the herbicide treatment and negative indicates plants that were sensitive to the herbicide.

<sup>2</sup>+ indicates hybridization signal on Southern blot; - indicates no hybridization signal on Southern blot. The cry34Ab1 gene probe hybridized to the expected internal T-DNA fragment of 1.915 kb, the cry35Ab1 gene probe hybridized to the expected internal T-DNA fragment of 2.607 kb, and the pat gene probe hybridized to a 3.4 kb border fragment consistent with a single intact T-DNA insertion as determined by Southern blot analysis.

on dry ice and were transferred to an ultralow freezer following collection. All samples were maintained frozen until processing. All leaf samples were uniquely labeled with the plant identifier and the date of harvest.

To confirm the expression of the Cry34Ab1 protein in event DAS-59122-7 and the absence of expression in the controls, leaf samples were collected from all event DAS-59122-7 and control plants, and screened for transgenic protein using lateral flow devices specific for Cry34Ab1 (Strategic Diagnostics, Inc., Newark, Del.). Leaf punches were taken from each plant and ground in a phosphate buffered saline solution with Tween 20 to crudely extract the protein. A strip device was dipped into the extract to determine the presence or absence of the Cry34Ab1 protein. The immunoassay results were used

## Example 3

Southern Blot Analysis of *Bacillus thuringiensis*  
Cry34/35Ab1 Maize Line DAS-59122-7

One gram quantities of leaf samples were ground under liquid nitrogen, and the genomic DNA was isolated using DNeasy® Plant Mini Kit (Qiagen, Valencia, Calif.) or using a standard Urea Extraction Buffer procedure. Following extraction, the DNA was visualized on an agarose gel to determine the DNA quality, and was quantified using Pico Green® reagent (Molecular Probes, Inc., Eugene, Oreg.) and spectrofluorometric analysis.

## US 7,956,246 B2

23

The 1 Kb DNA Ladder (Invitrogen, Carlsbad, Calif.) was used to estimate DNA fragment sizes on agarose gels.

Genomic DNA isolated from event DAS-59122-7 plants was digested with Nco I and electrophoretically separated, transferred to nylon membranes, and hybridized to the cry34Ab1, cry35Ab1 and pat gene probes using standard procedures known in the art. Blots were exposed to X-ray film for one or more time periods to detect hybridizing fragments and to visualize molecular weight standards. Images were then digitally captured by photographing X-ray films and/or by detection with a Lumi-Imager™ instrument (Roche, Indianapolis, Ind.). The sizes of detected bands were documented for each probe. Southern blot analysis was used as a means of verifying the presence of the insertion in the test plants and confirming that all plants from event DAS-59122-7 contained the same insertion as shown in Table 1. (Southern blots not shown.) Southern blot analysis indicated that event DAS-59122-7 contained a single insertion consisting of an intact copy of the T-DNA region from plasmid PHP17662, while the null segregants, as determined by the protein expression analysis did not hybridize to the gene probes. Further, event DAS-59122-7 plants expressing the two proteins exhibited identical hybridization patterns on Southern blots (data not shown). Specifically, the cry34Ab1 gene probe hybridized to the expected internal T-DNA fragment of 1.915 kb, the cry35Ab1 gene probe hybridized to the expected internal T-DNA fragment of 2.607 kb, and the pat gene probe hybridized to a 3.4 kb border fragment consistent with a single intact T-DNA insertion as determined by Southern blot results.

## Example 4

T-DNA Insert and Flanking Border Region  
Sequencing of *Bacillus thuringiensis* Cry34/35Ab1  
Maize Line DAS-59122-7

The T-DNA insert and flanking border regions were cloned from B.t. Cry34/35Ab1 event DAS59122-7 using PCR based methods as diagramed in FIGS. 2 and 3. Specifically, sequences bordering the 5' and 3' ends of the insert in event DAS-59122-7 were obtained using two genome walking techniques. The first walking method was essentially the method as described for the Universal Genome Walker Kit (BD Biosciences Clontech, Palo Alto, Calif.), and the second method was conducted according to the splinkerette protocol outlined in Devon et al., (1995) *Nucleic Acids Research* 23 (9):1644-1645, with modifications as described by Stover (2001), U. C. Irvine (personal communication).

Briefly, genomic DNA was digested with various restriction enzymes (Dra I, EcoR V, Pvu II, Sma I and Stu I for the Universal Genome Walker method and BamH I, EcoR I, Hind III, and Xba I for the splinkerette method) and then ligated to blunt-end adaptors for the Genome Walker method and to adaptors specific for the restriction enzyme used for the splinkerette method. The adaptors for both genome walking methods were designed to prevent extension of the 3' end of the adaptor during PCR and thus reduce or eliminate nonspecific amplification. The adaptor-ligated genomic DNA fragments were then referred to as genome walker libraries or splinkerette libraries, one library for each restriction enzyme. Libraries were prepared from genomic DNA isolated from three individual T1S2 plants of B.t. Cry34/35Ab1 event DAS-59122-7; plants DAS-59122-7 T1S2 1, DAS-59122-7 T1S2 2 and DAS-59122-7 T1S2 10, and from one Hi-II and one PH09B control plant.

24

Following construction of the libraries, nested PCR amplifications were completed to amplify the target sequence using Advantage™-GC Genomic PCR kit (BD Biosciences Clontech, Palo Alto, Calif.). The primary PCR amplification used one primer with identity to the adaptor and one gene specific primer. The adaptor primer will not amplify a product in the first cycle of the primary PCR and only products from the gene specific primer will be produced. Annealing and amplification from the adaptor primer only occurs after the complementary strand has been produced from the gene specific primer. Following primary PCR amplification, a secondary nested PCR reaction was performed to increase the specificity of the genomic PCR reactions. The nested primers consisted of gene-specific and adaptor-specific sequences internal to the respective primers used in the primary PCR.

For 5' flanking border sequences, nested PCR was initiated using primers specific to the 5' end of the inserted T-DNA along with primers complementary to the adaptor sequence ligated onto the digested DNA. Similarly, cloning of the 3' flanking border sequence started with a primer specific for the 3' end of the inserted T-DNA and a primer complementary to the adaptor sequence. DNA sequences internal to the T-DNA Right Border and Left Border sequences within the T-DNA region were used as the starting points for "walking out" to the maize genomic sequence, because they represented unique sequence (not homologous to endogenous maize genomic sequences) from which to anchor the genome walking primers.

The products produced by the nested PCR were analyzed by agarose gel electrophoresis (data not shown). Fragments visible in libraries prepared from one or more of the event DAS-59122-7 DNA samples and absent in libraries prepared from the Hi-II and PH09B genomic DNA samples were identified for further characterization. The identified PCR amplified fragments were separated by preparatory gel electrophoresis, isolated using a QIAquick Gel Extraction Kit (Qiagen), and sent directly for sequencing or cloned into a pGEM-T Easy plasmid vector using the pGEM-T Easy Vector System 1 (Promega Corp., Madison, Wis.) prior to DNA sequencing. Sequencing reactions were carried out with primers used for the nested PCR amplification or with primers specific for use with the pGEM-T Easy vector. The sequence obtained was used to design additional gene specific primers to continue "walking out" into the unknown maize genomic sequence. Multiple rounds of genome walking were performed until at least 500 bp of border sequence from the ends of the T-DNA insert were obtained.

To ensure validity of the flanking border sequences, additional event-specific PCR amplifications on genomic DNA from event DAS-59122-7 were performed. The amplified fragments were sequenced in order to further extend the region of sequence overlap from the T-DNA insert region into the 5' and 3' bordering genomic DNA. Primers, shown in Table 2, were designed based on the sequence obtained from the genome walking experiments to amplify a fragment spanning the unique junction of the T-DNA with the corn genomic DNA. Primer set 03-O-506/02-O-476 (SEQ ID NO: 10/SEQ ID NO:9) spanned the 5' junction and amplified a 313 bp fragment (from bp 2427 to bp 2739, see FIG. 1), and primer set 02-O-447/03-O-577 (SEQ ID NO: 8/SEQ ID NO:17) spanned the 3' junction and amplified a 754 bp fragment (from bp 9623 to bp 10376, see FIG. 1).

## US 7,956,246 B2

25

TABLE 2

Primer Sequences		
Primer Name	Sequence (5'-3')	Target Sequence Location (bp to bp) <sup>1</sup>
02-O-215	(SEQ ID NO: 1) GTGGCTCCTCAACGTTGCGGTTCTGTC	2743-2716
02-O-219	(SEQ ID NO: 2) CGTGCAAGCGCTCAATTCGCCCTATAGTG	9830-9858
02-O-227	(SEQ ID NO: 3) AATTGAGCGCTTGACGTTT	9846-9827
02-O-370	(SEQ ID NO: 4) AACCAACAAGACCGCCACACCTC	4871-4894
02-O-371	(SEQ ID NO: 5) GAGGTGGTCTGGATGGTGTAGGTCA	5187-5163
02-O-372	(SEQ ID NO: 6) TACAACCTCAAGTGGTTCCTCTTCCCGA	7017-7044
02-O-373	(SEQ ID NO: 7) GAGGTCTGGATCTGCATGATGCGGA	7897-7873
02-O-447	(SEQ ID NO: 8) AACCCTTAGTATGTATTGTATT	9623-9645
02-O-476	(SEQ ID NO: 9) CTCCTTCAACGTTGCGGTTCTGTCTAG	2739-2714
03-O-506	(SEQ ID NO: 10) TTTTGCAAAGCGAAGATTGATG	2427-2451
03-O-514	(SEQ ID NO: 11) GCGGGACAAGCGTTTACGTTT	2687-2709
03-O-542	(SEQ ID NO: 12) GACGGGTGATTATTTGATCTGCAC	1076-10742
03-O-543	(SEQ ID NO: 13) CATCTGAATCGTTCGCTTTGCAAAA	2451-2427
03-O-564	(SEQ ID NO: 14) CTACGTTTCAATGGAGCTCGACTGTC	2324-2299
03-O-569	(SEQ ID NO: 15) GGTCAAGTGGACACTTGGTCACTCA	10150-10174
03-O-570	(SEQ ID NO: 16) GAGTGAAGAGATAAGCAAGTCAAAG	10275-10299
03-O-577	(SEQ ID NO: 17) CATGTATACGTAAGTTTGGTGTGG	10376-10352
03-O-784	(SEQ ID NO: 18) AATCCACAAGATTGGAGCAAACGAC	2189-2213
67609	(SEQ ID NO: 36) CGTATTACAATCGTACGCAATTCAG	9862-9886
69240	(SEQ ID NO: 37) GGATAACAAACGGGACCATAGAAG	9941-9965

<sup>1</sup>Location in sequence of Event DAS-59122-7 (see FIG. 1). Bases 1 - 2593 = 5' border, bases 2594 - 9936 = T-DNA insert, bases 9937 - 11922 = 3' border.

For verification of the DNA sequence that inserted into the maize genome, PCR was performed to amplify, clone, and sequence the inserted T-DNA from event DAS-59122-7. PCR primer sets, (SEQ ID NO: 11/SEQ ID NO:5); (SEQ ID NO: 4/SEQ ID NO:7); and (SEQ ID NO: 6/SEQ ID NO:3) shown in Table 3 were used to amplify three overlapping fragments labeled 22I-1 (SEQ ID NO: 25), 22I-2 (SEQ ID NO: 26), and 22I-3 (SEQ ID NO: 27) representing sequence from the 5' region of the T-DNA running through to the 3' region of the

26

T-DNA insert from bp 2687 to bp 9846 for event DAS-59122-7 (see FIG. 1). PCR amplicon information is reported in Table 3 and primer sequences are listed in Table 2.

TABLE 3

PCR Primer and Amplicon Descriptions					
PCR Amplicon	Size (bp)	Target Sequence	Forward Primer	Reverse Primer	Location of PCR Amplicon (bp to bp) <sup>1</sup>
22I-1 (SEQ ID NO: 25)	2501	T-DNA insert	03-O-514 (SEQ ID NO: 11)	02-O-371 (SEQ ID NO: 5)	2687-5187
22I-2 (SEQ ID NO: 26)	3027	T-DNA insert	02-O-370 (SEQ ID NO: 4)	02-O-373 (SEQ ID NO: 7)	4871-7897
22I-3 (SEQ ID NO: 27)	2830	T-DNA insert	02-O-372 (SEQ ID NO: 6)	02-O-227 (SEQ ID NO: 3)	7017-9846
O784/O564 (SEQ ID NO: 28)	136	5' genomic border	03-O-784 (SEQ ID NO: 18)	03-O-564 (SEQ ID NO: 14)	2189-2324
O784/O543 (SEQ ID NO: 29)	263	5' genomic border	03-O-784 (SEQ ID NO: 18)	03-O-543 (SEQ ID NO: 13)	2189-2451
O569/O577 (SEQ ID NO: 30)	227	3' genomic border	03-O-569 (SEQ ID NO: 15)	03-O-577 (SEQ ID NO: 17)	10150-10376
O570/O542 (SEQ ID NO: 31)	492	3' genomic border	03-O-570 (SEQ ID NO: 16)	03-O-542 (SEQ ID NO: 12)	10275-10766
O784/O215 (SEQ ID NO: 32)	555	5' junction	03-O-784 (SEQ ID NO: 18)	02-O-215 (SEQ ID NO: 1)	2189-2743
O219/O577 (SEQ ID NO: 33)	547	3' junction	02-O-219 (SEQ ID NO: 2)	03-O-577 (SEQ ID NO: 17)	9830-10376
O506/O476 (SEQ ID NO: 34)	313	5' junction	03-O-506 (SEQ ID NO: 10)	02-O-476 (SEQ ID NO: 9)	2427-2739
O447/O577 (SEQ ID NO: 35)	754	3' junction	02-O-447 (SEQ ID NO: 8)	03-O-577 (SEQ ID NO: 17)	9623-10376
67609/69240 (SEQ ID NO: 38)	104	3' junction	67609 (SEQ ID NO: 36)	69240 (SEQ ID NO: 37)	9862-9965

<sup>1</sup>Location in sequence of Event DAS-59122-7 (see FIG. 1). Bases 1-2593 = 5' border, bases 2594-9936 = T-DNA insert, bases 9937-11922 = 3' border.

PCR GC2 Advantage™ Polymerase kit (BD Biosciences Clontech, Inc.) was used according to manufacturer's instructions to amplify the insert fragments (22I-1 (SEQ ID NO: 25), 22I-2 (SEQ ID NO: 26), and 22I-3 (SEQ ID NO: 27)). Briefly, a 50 L reaction contained 5' and 3' primers at a final concentration of 0.2 μM and 40 ng of genomic DNA. PCR reactions were set up in duplicate using genomic DNA preparation from plants DAS-59122-7 T1S2 1 and DAS-59122-7 T1S2 2. PCR conditions were as follows: initial denaturation at 95° C. for 1 min, followed by 35 cycles of 94°/95° C. for 30 sec, 55° C. for 30 sec, and 68° C. for 5 min, with final extension at 68° C. for 6 min. PCR amplification products were visualized under UV light, following electrophoresis through a 1% agarose gel in 1×TBE (89 mM Tris-Borate, 2 mM EDTA, pH 8.3) stained with ethidium bromide.

PCR fragments 22I-1 (SEQ ID NO: 25), 22I-2 (SEQ ID NO: 26), and 22I-3 (SEQ ID NO: 27) were purified by excising the fragments from 0.8% agarose gel in 1×TBE stained with ethidium bromide, and purifying the fragment from the agarose using a QIAquick Gel Extraction Kit (Qiagen). PCR fragments were cloned into a pGEM-T Easy plasmid vector using the pGEM-T Easy Vector System I (Promega Corp.). Cloned fragments were verified by miniprep of the plasmid DNA (QIAprep Spin Miniprep Kit, Qiagen) and

## US 7,956,246 B2

27

restriction digestion with Not I. Plasmid clones and/or purified PCR insert fragments were then sent for sequencing of the complete insert. Sequencing reactions were carried with primers designed to be specific for known T-DNA sequences or with primers specific for use with the pGEM-T Easy vector. Sigma-Genosys, Inc. (The Woodlands, Tex.) synthesized all PCR primers, which were used at a final concentration of 0.2-0.4  $\mu$ M in the PCR reactions.

PCR reactions with genomic DNA isolated from B.t. Cry34/35Ab1 events DAS-59122-7, DAS-45214-4, and DAS-45216-6, and unmodified control lines Hi-II and PH09B were used to confirm (1) the presence of maize genomic DNA in the sequenced border regions of event DAS-59122-7, and (2) event specific amplification across the junctions of the T-DNA insert and genomic DNA borders in event DAS-59122-7.

PCR primers designed to amplify the border sequence flanking the insert in event DAS-59122-7 were used to confirm the presence of those regions in unmodified control lines as well as in event DAS-59122-7. Two (2) sets of primers each, for the 5' and 3' borders (four (4) sets total) were tested. Primer sets 03-O-784/03-O-564 (SEQ ID NO: 18/SEQ ID NO: 14) and 03-O-784/03-O-543 (SEQ ID NO: 18/SEQ ID NO: 13) were used to amplify 136 bp and 263 bp fragments, respectively, from border sequence 5' to the T-DNA insert in event DAS-59122-7 (FIGS. 2 and 3). Similarly, primer sets 03-O-569/03-O-577 (SEQ ID NO: 15/SEQ ID NO: 17) and 03-O-570/03-O-542 (SEQ ID NO: 16/SEQ ID NO: 12) were used to amplify 227 bp and 492 bp fragments, respectively, from the 3' genomic border (FIGS. 2 and 3).

Primers designed to amplify fragments across the junction of the border sequence and T-DNA insert were used to establish event-specific PCR fragments for event DAS-59122-7. One primer set was selected for each of the two junctions. Primer set 03-O-784/02-O-215 (SEQ ID NO: 18/SEQ ID NO: 1) was designed to amplify a 555 bp fragment across the 5' junction, and primer set 02-O-219/03-O-577 (SEQ ID NO: 2/SEQ ID NO: 17) was designed for amplification of a 547 bp fragment at the 3' junction. A set of primers, IVR1(O197) (SEQ ID NO: 39) 5'-CCGCTGTATCACAAAGGGCTGGTACC-3' and IVR2(O198) (SEQ ID NO: 40) 5'-GGAGC-CCGTGTAGAGCATGACGATC-3', based on the endogenous maize invertase gene (Hurst et al., (1999) *Molecular Breeding* 5 (6):579-586), was used to generate a 226 bp amplification product as an internal positive control for all maize genomic DNA samples.

All PCR primers were synthesized by Sigma-Genosys, Inc. and used at a final concentration of 0.2-0.4  $\mu$ M in the PCR reactions. PCR primer sequences are listed in the Table 2. For PCR amplifications, Advantage<sup>TM</sup>-GC 2 PCR kit (BD Biosciences) was used according to manufacturer's instructions. Approximately 10-100 ng of genomic DNA template was used per 50  $\mu$ L PCR reaction. PCR conditions were as follows: initial template denaturation at 94° C. for 5 min, followed by 35 cycles of 95° C. for 1 minute, 60° C. for 2 minutes, and 72° C. for 3 min, with final extension at 72° C. for 7 min. The PCR amplification products were visualized under UV light following electrophoresis through a 1% agarose gel with 1 $\times$ TBE and ethidium bromide.

Sequence data obtained for the T-DNA insert and border regions of event DAS-59122-7 was reviewed and assembled using Seqman II<sup>TM</sup> software Version 4.0.5 (DNASTar, Inc., Madison, Wis.). The 5' and 3' border sequences flanking the insert present in event DAS-59122-7 were used for homology searching against the GenBank public databases in order to further characterize the site of insertion in the maize genome. Analysis to identify open reading frames in the junction

28

regions between the flanking borders and T-DNA insert in event DAS-59122-7 was conducted using Vector NTI 8.0 (InforMax<sup>TM</sup>, Inc., Frederick, Md.).

In total, 11922 bp of sequence from genomic DNA of event DAS-59122-7 was confirmed (see FIG. 1). At the 5' end of the T-DNA insert, 2593 bp of flanking border sequence was identified, and 1986 bp of flanking border sequence was obtained on the 3' end from fragments derived from genome walking experiments. A total of 7160 bp of the T-DNA insert was cloned and sequenced using PCR primer sets designed to amplify three overlapping fragments labeled 221-1 (2501 bp) (SEQ ID NO: 25), 221-2 (3027 bp) (SEQ ID NO: 26), and 221-3 (2830 bp) (SEQ ID NO: 27) representing sequence from the 5' region of the T-DNA running through to the 3' region of the T-DNA insert for event DAS-59122-7 from bp 2687 to bp 9846 (see FIG. 1). The remainder of the T-DNA insert region was sequenced from two PCR fragments, O506/O476 (SEQ ID NO: 10/SEQ ID NO: 9) and O447/O577 (SEQ ID NO: 8/SEQ ID NO: 17) that spanned the 5' and 3' junctions, respectively, of the T-DNA insert with corn genomic DNA. Primers used were designed based on the sequence obtained from the genome walking experiments to amplify a fragment spanning the unique junction of the T-DNA with the corn genomic DNA. Primer set 03-O-506/03-O-476 (SEQ ID NO: 10/SEQ ID NO: 9) spanned the 5' junction and amplified a 313 bp fragment (from bp 2427 to bp 2739) and primer set 03-O-447/03-O-577 (SEQ ID NO: 8/SEQ ID NO: 17) spanned the 3' junction and amplified a 754 bp fragment (from bp 9623 to bp 10376). Combined, a total of 7343 bp of the T-DNA insert in event DAS-59122-7 was cloned and sequenced (from bp 2594 to bp 9936, see FIG. 1) and compared to the sequence of the transforming plasmid, PHP17662. Two nucleotide differences at bp 6526 and bp 6562 were observed in the non-translated wheat peroxidase promoter region of the T-DNA insert (see FIG. 1). Neither of the observed base changes affected the open reading frame composition of the T-DNA insert. Both the 3' and 5' end regions of the T-DNA insert were found to be intact, except for deletion of the last 22 bp at the 5' end and 25 bp at the 3' end encompassing the Right and Left T-DNA Border regions, respectively. While T-DNA border sequences are known to play a critical role in T-DNA insertion into the genome, this result is not unexpected since insertions are often imperfect, particularly at the Left T-DNA Border (Tinland (1996) *Trends in Plant Science* 1(6): 178-184).

BLAST (Basic Local Alignment Search Tool) analysis of the genomic border regions of event DAS-59122-7 showed limited homology with publicly available sequences (Release 138.0 GenBank, Oct. 25, 2003). Analysis of the 5' border region found two areas with significant homology to maize genomic and EST (Expressed Sequence Tag) sequences. The first area encompasses 179 bp (bp 477 to bp 655 of the border sequence) and displays similarity to several molecular markers, chromosomal sequences, and consensus sequences obtained by alignment of various ESTs. The second area occurs at bp 1080 to bp 1153 (74 bp) of the 5' border sequence, and shows similarity to a number of different maize ESTs and genomic sequences. The 3' border region also had two small non-contiguous regions of similarity to plant DNA sequences. The inner 3' region of 162 bp (bp 9954 to bp 10115) showed similarity to the 3' untranslated end of two genomic *Zea mays* alcohol dehydrogenase (adh1) genes as well as to several EST consensus sequences. A smaller region (57 bp) in the middle of the 3' border (bp 10593 to bp 10649) showed similarity to non-coding regions from multiple maize genomic sequences.

Overall, no homologous regions greater than 179 base pairs were identified in either of the genomic border



## US 7,956,246 B2

29

sequences, nor was more than one homologous region from the same known sequence found. Individual accessions displaying similarity to the event DAS-59122-7 border sequences were examined to determine if the insertion in event DAS-59122-7 occurred in a characterized protein coding sequence. None of the regions of similarity occurred within any known protein coding sequences. Local alignment of the entire transformation plasmid sequence, PHP17662, with the event DAS-59122-7 border sequences showed no significant homologies, indicating that the border regions flanking the T-DNA insert did not contain fragments of the transforming plasmid. Therefore, identification and characterization of the genomic sequence flanking the insertion site in event DAS-59122-7 was limited due to the absence of significant regions of homology to known sequences.

The 5' and 3' junction regions between the maize genomic border sequence and the T-DNA insert in event DAS-59122-7 were analyzed for the presence of novel open reading frames. No open reading frames of significant size (>100 amino acids) were identified in the 5' or 3' border junction regions, indicating that no novel open reading frames were generated as a result of the T-DNA insertion. Additionally, the homology searches did not indicate the presence of endogenous maize open reading frames in the border regions that might have been interrupted by the T-DNA insertion in B.t. Cry34/35Ab1 event DAS-59122-7.

## Example 5

## PCR Primers

DNA event specific primer pairs were used to produce an amplicon diagnostic for DAS-59122-7. These event primer pairs include, but are not limited to, SEQ ID NO: 18 and SEQ ID NO: 1; SEQ ID NO: 2 and SEQ ID NO: 17; SEQ ID NO: 10 and SEQ ID NO: 9; and SEQ ID NO: 8 and SEQ ID NO: 17; and SEQ ID NO: 36 and SEQ ID NO: 37. In addition to these primer pairs, any primer pair derived from SEQ ID NO: 21 and SEQ ID NO: 22 that when used in a DNA amplification reaction produces a DNA amplicon diagnostic for DAS-59122-7 is an embodiment of the present invention. Any modification of these methods that use DNA primers or complements thereof to produce an amplicon DNA molecule diagnostic for DAS-59122-7 is within the ordinary skill of the art. In addition, control primer pairs, which include 1VR1

30

(O197)/1VR2(O198) (SEQ ID NO: 39/SEQ ID NO: 40) for amplification of an endogenous corn gene are included as internal standards for the reaction conditions.

The analysis of plant tissue DNA extracts to test for the presence of the DAS-59122-7 event should include a positive tissue DNA extract control (a DNA sample known to contain the transgenic sequences). A successful amplification of the positive control demonstrates that the PCR was run under conditions that allow for the amplification of target sequences. A negative, or wild-type, DNA extract control in which the template DNA provided is either genomic DNA prepared from a non-transgenic plant, or is a non-DAS-59122-7 transgenic plant, should also be included. Additionally a negative control that contains no template corn DNA extract will be a useful gauge of the reagents and conditions used in the PCR protocol.

Additional DNA primer molecules of sufficient length can be selected from SEQ ID NO: 21 and SEQ ID NO: 22 by those skilled in the art of DNA amplification methods, and conditions optimized for the production of an amplicon diagnostic for event DAS-59122-7. The use of these DNA primer sequences with modifications to the methods shown in these Examples are within the scope of the invention. The amplicon wherein at least one DNA primer molecule of sufficient length derived from SEQ ID NO: 21 and SEQ ID NO: 22 that is diagnostic for event DAS-59122-7 is an embodiment of the invention. The amplicon wherein at least one DNA primer of sufficient length derived from any of the genetic elements of PH17662A that is diagnostic for event DAS-59122-7 is an embodiment of the invention. The assay for the DAS-59122-7 amplicon can be performed by using a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler, or by methods and apparatus known to those skilled in the art.

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

## SEQUENCE LISTING

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US 7,956,246 B2

31

32

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US 7,956,246 B2

33

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US 7,956,246 B2

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36

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US 7,956,246 B2

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US 7,956,246 B2

39

40

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## US 7,956,246 B2

41

42

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gagtcgtcct gctgctcat gtcacctcga gtcccgccac gacctcagtg cttgttcttg	2040
ttggagccac ctctctcgga cgatcgccaa agacggataa ggccgaagcc gtcacttcag	2100
accgcgtca tgcgcgtag cagactccta catagcaggg ccagggtatg tggacctttg	2160
caagtttagg attggaacca gcgaccagaa tccacaagat tggagcaaac gacaaaaaat	2220
tcacaaggat tggcggtgta cattgccagc gcgggatcgc atgcggcggc ggcggccggg	2280
gcgagcacgg gagcaggcga cagtcgagct ccattggaac gtagaaatac ttaagggcaa	2340
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caattggaag atgttatgaa tcttgttttt gcaaaagcaa cgattcagat ggcaaaacta	2460
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gcacctgtga ttggctcata aaaattcttg gagggacgga agaaagagtg aagggataag	2580
caagtaaaag cgctcaaaca ctgatagttt aaactgaagg cgggaaacga caatctgatc	2640
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tttactgttg gaactgacag aaccgcaacg ttgaaggagc cactcagcaa gcttactagt	2760
agcgctgttt aaacgctctt caactggaag agcgggtacc cggaccgaag cttgcatgcc	2820
tgcagtgcag cgtgaccgg tcgtgccct ctctagagat aatgagcatt gcatgtctaa	2880

US 7,956,246 B2

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gttataaaaa attaccacat attttttttg tcacacttgt ttgaagtgca gtttatctat 2940
ctttatacat atattttaaac ttactctac gaataatata atctatagta ctacaataat 3000
atcagtgttt tagagaatca tataaatgaa cagttagaca tggcttaaag gacaattgag 3060
tattttgaca acaggactct acagttttat ctttttagtg tgcattgtgt ctcctttttt 3120
tttgcaaata gcttcaccta tataataact catccatttt attagtacat ccatttaggg 3180
tttaggggta atgggtttta tagactaatt ttttttagtac atctatttta ttctatttta 3240
gcctctaaat taagaaaact aaaactctat tttagttttt ttatttaata atttagatat 3300
aaaaatagaat aaaataaagt gactaaaaat taaacaaata ccctttaaga aattaaata 3360
actaaggaaa catttttctt gtttcgagta gataatgccg gcctgttaaa cgcgctcgac 3420
gagtctaacg gacaccaacc agcgaaccag cagcgctcgc tgggccaag cgaagcagac 3480
ggcacggcat ctctgtcgt gcctctggac ccctctcgag agttccgctc caccgttggg 3540
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<210> SEQ ID NO 22
<211> LENGTH: 2987
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Sequence that represents part of the
PHI17662A insert as well as flanking sequence 3' to the
insert.
```

<400> SEQUENCE: 22

```
ctcggagag gagaccagt gagattaggc cagctacagc agctgatatg gccgcggttt 60
gtgatatcgt taaccattac attgagacgt ctacagtga ctttaggaca gagccacaaa 120
caccacaaga gtggattgat gatctagaga ggttgcaaga tagataccct tggttggttg 180
ctgaggttga ggggtgtgtg gctggtattg cttacgctgg gccctggaag gctaggaacg 240
cttacgattg gacagttgag agtactgttt acgtgtcaca taggcacaa aggttggggc 300
taggatccac attgtacaca catttgctta agtctatgga ggcgcaagg ttaagtctg 360
tggttgctgt tataggcctt ccaaacgac catctgttag gttgcatgag gctttgggat 420
acacagcccg ggttacattg cgcgcagctg gataacgca tggtggaagg catgatgtt 480
gtttttggca aagggatttt gagttgccag ctctccaag gccagttagg ccagttaccc 540
agatctgagt cgacctgcag gcatgccgc tgaatcacc agtctctctc tacaatcta 600
tctctctcta taataatgtg tgagtagtcc ccagataagg gaattagggt tcttatagg 660
tttcgctcat gtgttgagca tataagaaac ccttagtatg tatttgtatt tgtaaaatac 720
ttctatcaat aaaatttcta attcctaaaa ccaaatcca gggcgagctc ggtaccggg 780
gatcctctag agtcgacctg caggcatgcc cgcggatgc gatgggccc gccgaagct 840
tcggtcggg ccacgtggc ctcttgcctc tcaggatgaa gagctatgt taaacgtgca 900
agcgtcctat tcgcccata gtgagtcgta ttacaatcgt acgcaattca gtacattaaa 960
aacgtccgca atgtgttatt aagttgtcta agcgtcaatt tttccctct atggteccgt 1020
ttgtttatcc tctaaattat ataaccagc ttaataaagt taagagacaa acaacaaca 1080
cagattatta aatagattat gtaactaga tacctagatt atgtaatcca taagtagaat 1140
atcaggtgct tatataatct atgagctcga ttatataatc ttaaaagaaa acaaacagag 1200
cccctataaa aaggggtcaa gtggacactt ggtcactcat ttaatccctc cctctcctc 1260
tttatccctc tttttggtgt attcaccat agtggtgtgc acctgtgatt ggctcgtaaa 1320
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US 7,956,246 B2

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46

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aattcttgga cggatggaag agtgaagaga taagcaagtc aaagaaaagt aacaacgaag	1380
cttcacacgc tacaaatttt ggcccaactg gttgcaccag caccaaactt acgtatacat	1440
gattatctct gtttccctca tttcgaagaa aaaaacgggt ttcaaaaccc actgctttca	1500
ggagtaaaaa aagataataa tctgaacat tgcttcacc ttggccctta tttggttacg	1560
ttgcaattca cccaatcca catgtggatt gagatggatt gcagtgtagc tagacaaacc	1620
cttaggccct gtttgcatag gaatacacca ggaattattc cagctaatca aaatttatat	1680
aaatgagaga aacaattcgg ataggaattg ttccaggact tcattctgca gtaaccgaac	1740
ggccctctaa tccaccccaa tacacgtgga ttggagtgga ttgaggtaca gccaaacaag	1800
gcctaagtgc agatcaaata aatcacccgt catattcttc tacctacaaa aacagcaata	1860
aacacctgaa tgaagtctca atttgacag ttaggttagg atgaaatag ttacctctc	1920
atggtcagta actcttggca cacaactca catgtaatcg atgtaccact tggctcttgc	1980
ctgaaaccca atacatcttt agcataagaa taatattatg atggcaaggc atgatcacca	2040
gcactccttt attgtttagt aagtctatca ctcccaaaa caattcaaata gaacagagat	2100
gcattgcccc caatgaattc tatttcaatt agccggaaaa ttctacttca tcagaagcat	2160
ccaaattgcc agcatcccta ctagactgac catgaccagg ctgccgcaga tgctctttt	2220
tctgtctct cctctttgac ttgagtttct cttcaagatc cctcacccca cgtctcttat	2280
acatcttaaa gctaactgt ctctctctcg ccatcttct aaccttctca gtaatctcag	2340
cagcaatctg acggttgtag aacttcttca gcccttctc caactttgca aatgtgtcag	2400
gctgtggcat cagtcctgcc tctagcatgt ctaagcaata caggcaggcc tccttgacat	2460
gtttcttcgc aaacagtgca tgaatccaga tagtccatgc actcacattg agctcacagc	2520
ctttgtctac aatacatctt caaacatcct ttgcaagctc aagtttctca tctctgacca	2580
acgcattgag gaggtccttc agcaccccat attgcggtac cacaaagagc cccctcccaa	2640
ccatgtcttt aaaataacta catgcctcaa tcagcaaac ctgcccaaca aggccactca	2700
ccacgatagc aaatgtatcg accacaggac tgagccacgc actttccatc tcattccaca	2760
atgtcatggc ttgcttggtc tccccagcc tgcaggccaa ccgaatcacc acattgtata	2820
tcttgagatc tgggtggacac cggcactccc gcctcctctc catcagctcc aagcactcct	2880
caagctgctc cttcttctcg tgtgtacaa agaaacctg gtacacggca gcgtccaccc	2940
gcaggccatc cctcgacata gcaccaaga actcgtaccc ctgggat	2987

&lt;210&gt; SEQ ID NO 23

&lt;211&gt; LENGTH: 11922

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: The sequence represents the complete sequence of the insert and flanking regions of event DAS 59122-7.

&lt;400&gt; SEQUENCE: 23

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ggtgcggaca tggggcaacc tgcgcagcta acgcagggat ccacacgacc accaacgaag	120
ccaagcccg gcacgtcccc aggcagggtg ggccctggtt ccaccagcgg atcatgcag	180
tgaagcggg acggagagac aagccgaggg cgcgggtggg aatggcgtcc gggaggacga	240
gtggaggaga agaattctaga ggcatcgaga ttcgagaagc cgacggagac aagattcgtg	300
tggggggaga caaacccgga ggctgagcgc cgttgatatg ggatcagacg gtgtggataa	360

## US 7,956,246 B2

47

48

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aaaaagtgac gttgatagaa cgtctggcca gtgaaaaaac aaaacaactc caacaaaata	420
ctttaaaagc tcttatacc ctaaatgtagg ggatcaaaca cgtctctaca ctatttagca	480
gcgctctcta aatgatccctc taaattttaga gaacgctact agattctcta tatatagttt	540
ctctaaacga tcttttatcc atttaaatc tttaaataac cggtttaaca aaactaaaat	600
atatacaata catttgagag tatgacaaat acgtatgtat aaaaaataaa aataaaataa	660
tgtattagtc tactttgaat cttcttttct tcataatata atgatgtata gctctcatgt	720
gcgttgagaa aaaagtttaga gctagacgtt taatgtgtag tgacagctct cgacgaaatc	780
tcctaatga gatgaattac tggaggttcc atcagaaagt cccctgaaaa gaggcattta	840
tttagtttag tcagcaattt ctgggaacac aaatattctt ttgttatcac cactattaaa	900
aatctatggt tataacttat aataacatga aaaaaataatt tagcatccca tatatataaa	960
aactgaagga agccatatat actaacataa gttaggagaa actaagaagg ttgtgcaaag	1020
cttgcaactgc tccaaaatac tgcaacaac cactctctc taccaaccaa agaaactcat	1080
gtactcctc cgttcttttt tatttgctgc attttagttt aaaaatgaac tagcagtcga	1140
caaatattcg agaacagata tagtatatac taacataact taggagatac taagaaagtt	1200
gcgcagagct ttcactgttc caaattactg caaagcctct cccctctgcc agtacatcta	1260
cgagatgttt cagttaaaca aagattcaga caagtgatga gccacttctt gtcatagatt	1320
gtgtgggtcaa ccaaccatt gatgccacgg tttttgtgca tccatgcttt tgtattaaaa	1380
catcagttat gtttaccatg tccgatatgc tctacataat gacaatcaac ttggtgttca	1440
ttatatttac aatgttagga atttcaatag ctacgaacac ttcaatagaa gtgcctttgt	1500
gggatcacct taatgtgttg ttgatgtaag gagaagaatc ttaatttact cttgctaaat	1560
ttgaactaca caaaaccact gcactgagga ttgtcctaata aaattactgc tcatacacgt	1620
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caatcattca ttttagttat ttatttctta gttatccact tgaagattta catacatttg	1740
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gcaattagtg ctatgaatcg cgtttaagcg ctgcaaaatc atggctgggg cttcgtctc	1980
gagtcgtcct gctgctgat gtcacctcga gtcccgacc gacctcagtg cttgttcttg	2040
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caagtttagg attggaacca gcgaccagaa tccacaagat tggagcaaac gacaaaaaat	2220
tcacaaggat tggcggtcta cattgccagc gcgggatcgc atgcggcggc ggcggccggg	2280
gcgagcacgg gagcaggcga cagtcgagct ccattggaac gtagaaatac ttaagggcaa	2340
ggtctccaaa tacttgaaaa aataggaaaa agaagaaaat acatgaaatg atattgaaat	2400
caattggaag atgttatgaa tcttgttttt gcaaagcgaa cgattcagat ggcaaaacta	2460
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gcacctgtga ttggctcata aaaattcttg gagggacgga agaagagtg aagggataag	2580
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## US 7,956,246 B2

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aaaaatagaat aaaataaagt gactaaaaat taaacaaata ccctttaaga aattaaanaa	3360
actaaggaaa catttttctt gtttcgagta gataatgccg gcctgttaaa cgcgcgcgac	3420
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catcgacgtg aacaacaaga ccggccacac cctccagctg gaggacaaga ccaagctcga	4920
cggcggcagg tggcgcacct ccccgaccaa cgtggccaac gaccagatca agacctcgt	4980
ggccgaatcc aacggcttca tgaccggcac cgagggcacc atctactact caattaatgg	5040
cgaggccgag atcagcctct acttcgacaa ccggttcgcc ggctccaaca aatacgacgg	5100
ccactccaac aagtccagat acgagatcat caccagggc ggctccggca accagtcca	5160

## US 7,956,246 B2

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cgtagacctac accatccaga ccacctctc cegctacggc cacaagtcct gagtcacgag	5220
tcacgagtcg gttacacacg acttgctccat cttctggatt ggccaactta attaatgtat	5280
gaaataaaag gatgcacaca tagtgacatg ctaataccta taatgtgggc atcaaagttg	5340
tgtgttatgt gtaattacta gttatctgaa taaaagagaa agagatcatc catatttctt	5400
atcctaaatg aatgtcacgt gtctttataa ttctttgatg aaccagatgc atttcattaa	5460
ccaaatccat atacatataa atattaatca tatataatta atatcaattg ggtagcaca	5520
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aaaatgtcat ataaaacatt tggtgtctct tagttaggcc tgatcgtaga acgaaatgct	6060
cgtgtagcgg ggctacgagc ctatgacgca ataactctgg tttgccggcc cggagtcgct	6120
tgacaaaaaa aagcatgtta agtttattta caattcaaaa cctaacatat tatattccct	6180
caaagcaggt tcacgatcac acctgtacct aaaaaaaca tgaagaatat attactccat	6240
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ccctattgcc ggaaaaaaa tgcagggcag gtgttgcccg tagcgattta agcacttaag	6480
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ggatgccggg tcgacgtcga tcgtcagcca tcatagacca atcgaccatc tgttatggat	6660
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ccaacaaggt gtacgagatc agcaaccacg ccaacggcct ctacgcggcc acctacctct	6960
ccctcgacga ctccggcgtg tccctcatga acaagaacga cgcagacatc gacgactaca	7020
acctcaagtg gtctctcttc ccgatcgagc acgaccagta catcatcacc tctacgcgg	7080
ccaacaactg caaggtgtgg aacgtgaaca acgacaagat taatgtgtca acctactcct	7140
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ccgacaacgg caaggtgtct accgcggcga ccggccaggc cctcggcctc atccgcctca	7260
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ccggcaacat cgacaacggc acctccccgc agctcatggg ctggaccctc gtgcccgtgca	7440
tcacgttgaa cgaaccgaac atcgacaaga acaccagat caagaccacc ccgtactaca	7500
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## US 7,956,246 B2

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tcaacaccct cggcttcocag atcaacatcg acagcggcat gaagttcgac atcccggagg	7680
tggcgggcgg tacgcacgag atcaagaccc agctcaacga ggagctcaag atcgagtatt	7740
cacatgagac gaagatcatg gagaagtacc aggagcagtc cgagatcgac aaccgcaccg	7800
accagtcctat gaactccatc ggcttctctc ccatcacctc cctggagctc taccgctaca	7860
acggctccga gatccgcata atgcagatcc agacctccga caacgacacc tacaacgtga	7920
cctctctacc gaaccaccag caggccctgc tgcgtctgac caaccactcc tacgaggagg	7980
tggaggagat caccaacatc ccgaagtcca cctcaagaa gctcaagaag tactacttct	8040
gagtcagtga tcatgagtca gttaacctag acttgctcat cttctggatt ggccaactta	8100
attaatgtat gaaataaaag gatgcacaca tagtgacatg ctaatacta taatgtgggc	8160
atcaaagtgt tgtgttatgt gtaattacta gttatctgaa taaaagagaa agagatcatc	8220
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US 7,956,246 B2

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&lt;223&gt; OTHER INFORMATION: This sequence represents the DNA molecule used to transform maize line DAS59122-7 and represents insert PHI 17662A.

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&lt;222&gt; LOCATION: (1)...(25)

&lt;223&gt; OTHER INFORMATION: T-DNA right border

&lt;221&gt; NAME/KEY: misc\_feature

## US 7,956,246 B2

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&lt;222&gt; LOCATION: (7366)...(7390)

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## US 7,956,246 B2

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## US 7,956,246 B2

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US 7,956,246 B2

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tgggcggtcg ttctagatcg gagtagaatt ctgtttcaaa ctacctggtg gattttattaa 1560
ttttggatct gtatgtgtgt gccatacata ttcatagtta cgaattgaag atgatggatg 1620
gaaatatcga tctaggatag gtatacatgt tgatgcgggt tttactgatg catatacaga 1680
gatgcttttt gttcgtctgg ttgtgatgat gtggtgtggt tgggcggctg ttcattcgtt 1740

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## US 7,956,246 B2

65

66

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```
ctagatcgga gtagaatact gtttcaaact acctggtgta tttattaatt ttggaactgt 1800
atgtgtgtgt catacatctt catagttacg agtttaagat ggatggaaat atcgatgtag 1860
gatagggtata catgttgatg tgggttttac tgatgcatat acatgatggc atatgcagca 1920
tctattcata tgctctaacc ttgagtacct atctattata ataacaagt atgttttata 1980
attattttga tcttgatata cttggatgat ggcatatgca gcagctatat gtggattttt 2040
ttagccctgc cttcatacgc tatttatttg cttggtactg tttcttttgt cgatgctcac 2100
cctgttgttt ggtgttactt ctgcaggteg actctagagg atccacacga caccatgtcc 2160
gcccgcgagg tgcacatcga cgtgaacaac aagaccggcc acaccctcca gctggaggac 2220
aagaccaagc tcgacggcgg caggtggcgc acctccccga ccaacgtggc caacgaccag 2280
atcaagacct tcgtggccga atccaacggc ttcattgaccg gcaccgaggg caccatctac 2340
tactcaatta atggcgaggc cgagatcagc ctctacttcg acaaccggtt cgccggtccc 2400
aacaatacgc acggccactc caacaagtcc cagtacgaga tcatacccca gggcggtccc 2460
ggcaaccagt cccacgtgac ctacaccatc cagaccacct c 2501
```

&lt;210&gt; SEQ ID NO 26

&lt;211&gt; LENGTH: 3027

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: PCR Amplicon 22I-2.

&lt;400&gt; SEQUENCE: 26

```
aacaacaaga cgggccacac cctccagctg gaggacaaga ccaagctcga cggcggcagg 60
tggcgccacct ccccgaccaa cgtggccaac gaccagatca agaccttcgt ggccgaatcc 120
aacggcttca tgaccggcac cgagggcacc atctactact caattaatgg cgaggccgag 180
atcagcctct acttcgacaa ccggttcgcc ggctccaaca aatacgacgg ccaactccaa 240
aagtcccatg acgagatcat caccagggc ggctccgcca accagtccca cgtgacctac 300
accatccaga ccacctctc cgcgtacggc cacaagtcct gagtcagtag tcatgagtca 360
gttaacctag acttgccat cttctggatt ggccaactta attaatgtat gaaataaaag 420
gatgcacaca tagtgacatg ctaatcacta taatgtgggc atcaaagttg tgtgttatgt 480
gtaattacta gttatctgaa taaaagagaa agagatcatc catatttctt atcctaaatg 540
aatgtcacgt gtctttataa ttctttgatg aaccagatgc atttcattaa ccaaatccat 600
atacatataa atattaatca tatataatta atatcaattg ggtagcaaa acaaatctag 660
tctaggtgtg ttttgogaat ggggcgcgg accgaattgg ggatctgcat gaaagaaact 720
gtcgcactgc tgaaccgcac cttgtcactt tcacgaaca cgacctgtgc ccaagatgac 780
gggtgctcgg tctaagttag gctgaattgc cttggacaga agcggactcc ctacaattag 840
ttaggccaaa cgggtgcacc atgtgtagct cggggtcgg gctgtatcgc catctgcaat 900
agcatccatg gagctcgttc catgtagttg gagatgaacc aatgatcggg cgtgtggacg 960
tatgttcctg tgactccga tagtagagta cgtgttagct ctttcattgt gcaagtgaag 1020
tttgtgttgg tttaattacc cctacgttag ttgcgggaca ggagacacat catgaattta 1080
aaggcgatga tgtcctctcc tgtaattgta ttcttttgat gtgatgaatc aaaatgtcat 1140
ataaaacatt tgttgcctct tagttaggcc tgatcgtaga acgaaatgct cgtgtagcgg 1200
ggctacgagc ctatgacgca ataacactgg tttgccggcc cggagtcgct tgacaaaaaa 1260
aagcatgtta agtttattta caattcaaaa cctaacatat tatattccct caaagcaggt 1320
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US 7,956,246 B2

67

68

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tcacgatcac acctgtacct aaaaaaaca tgaagaatat attactccat tattatgaga 1380
tgaaccactt ggcaagagtg gtaagctata taaaaaatg aacattatta cgagatgtta 1440
tatgccatta tattgattcg aagatatatg tttctttctc ccacgggcac ctaacggata 1500
catgataagg ccaaggcaga tcacgggaaa ttattcgaat acatgttacg ccctattgcc 1560
ggaaaaaaaa tgcagggcag gtgttgccg tagcgattta agcacttaag ctggagggtg 1620
ccacacttgg atgcaagcgt ctgaccttc taaaacatcg gcggctttgt ccgtatccgt 1680
atccctatc cgacatctag ctggccacac gacggggctg ggagatcgt ggatgccggg 1740
tcgagctcga tcgtcagcca tcatagacca atcgaccatc tgttatggat gcttgctagc 1800
tagactagtc agacataaaa tttggatact ttctcccaac tgggagacgg ggactgatgt 1860
gcagctgcac gtgagctaaa tttttcccta taaatatgca tgaataactg cattatcttg 1920
ccacagccac tgccacagcc agataacaag tgcagctggt agcacgcaac gcatagctct 1980
ggacttgtag ctaggtagcc aaccggatcc acacgacacc atgctcgaca ccaacaaggt 2040
gtacgagatc agcaaccacg ccaacggcct ctacgccgcc acctacctct ccctcgacga 2100
ctcggcgtg tccctcatga acaagaacga cgacgacatc gacgactaca acctcaagt 2160
gttctctctc ccgatcgacg acgaccagta catcatcacc tcttacgccg ccaacaactg 2220
caaggtgtgg aacgtgaaca acgacaagat taatgtgtca acctactcct ccaccaactc 2280
catccagaag tggcagatca aggccaacgg ctctccttac gtgatccagt ccgacaacgg 2340
caaggtgctc accgccggca ccggccaggc cctcggcctc atccgcctca ccgacgagtc 2400
ctccaacaac ccgaaccagc aatggaacct gacgtccgtg cagaccatcc agtcccgcga 2460
gaagccgatc atcgacacca agctcaagga ctaccgaag tactccccga ccggcaacat 2520
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gtaccagtac tggcagaggg ccgtgggctc caacgtcgcg ctccgccgcg acgagaagaa 2700
gtcttacacc tacgagtggg gcaccgagat cgaccagaag accaccatca tcaacacct 2760
cggtctccag atcaacatcg acagcggcat gaagttcgac atcccgaggg tgggcggcgg 2820
taccgacgag atcaagacc agctcaacga ggagctcaag atcgagtatt cacatgagac 2880
gaagatcatg gagaagtacc aggagcagtc cgagatcgac aaccggaccg accagtccat 2940
gaactccatc ggcttctcga ccatcacctc cctggagctc taccgctaca acggctccga 3000
gatccgcatc atgcagatcc agacctc 3027

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<210> SEQ ID NO 27
<211> LENGTH: 2830
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR Amplicon 22I-3.

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<400> SEQUENCE: 27

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tacaacctca agtggttcct cttcccgatc gacgacgacc agtacatcat cacctctac 60
gccgccaaca actgcaaggt gtggaacgtg aacaacgaca agattaatgt gtcaacctac 120
tcctccacca actccatcca gaagtggcag atcaaggcca acggctcctc ctacgtgac 180
cagtcggaca acggcaaggt gctcacggcc ggacccggcc aggccctcgg cctcatccgc 240
ctcaccgacg agtcctccaa caaccggaac cagcaatgga acctgacgtc cgtgcagacc 300
atccagctcc cgcagaagcc gatcatcgac accaagctca aggactaacc gaagtactcc 360

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## US 7,956,246 B2

69

70

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ccgaccggca	acatcgacaa	cggcacctcc	cgcagctca	tgggctggac	cctcgtgccg	420
tgcatcatgg	tgaacgaccc	gaacatcgac	aagaacaccc	agatcaagac	caccccgtac	480
tacatcctca	agaagtacca	gtactggcag	agggccgtgg	gctccaacgt	cgcgctccgc	540
ccgcacgaga	agaagtccta	cacctacgag	tggggcaccc	agatcgacca	gaagaccacc	600
atcatcaaca	ccctcggcct	ccagatcaac	atcgacagcg	gcatgaagtt	cgacatcccg	660
gaggtgggcg	gcggtaccga	cgagatcaag	accagctca	acgaggagct	caagatcgag	720
tattcacatg	agacgaagat	catggagaag	taccaggagc	agtccgagat	cgacaacccg	780
accgaccagt	ccatgaacte	catcggtctc	ctcaccatca	cctccctgga	gctctaccgc	840
tacaacggct	ccgagatccg	catcatgcag	atccagacct	ccgacaacga	cacctacaac	900
gtgacctcct	acccgaacca	ccagcaggcc	ctgctgctgc	tgaccaacca	ctcctacgag	960
gaggtgggag	agatcaccaa	catcccgaag	tccaccctca	agaagctcaa	gaagtactac	1020
ttctgagtca	tgagtcatga	gtcagttaac	ctagacttgt	ccatcttctg	gattggccaa	1080
cttaattaat	gtatgaaata	aaaggatgca	cacatagtga	catgctaata	actataatgt	1140
gggcatcaaa	gttgtgtgtt	atgtgtaatt	actagttatc	tgaataaaaag	agaaagagat	1200
catccatatt	tcttatcccta	aatgaatgtc	acgtgtcttt	ataattcttt	gatgaaccag	1260
atgcatttca	ttaaccaaat	ccatatacat	ataaatatta	atcatatata	attaatatca	1320
attgggttag	caaaacaaat	ctagtctagg	tgtgttttgc	gaattcccat	ggagtcaaag	1380
attcaaatag	aggacctaac	agaactcgcc	gtaaagactg	gcgaacagtt	catacagagt	1440
ctcttacgac	tcaatgacaa	gaagaaaatc	ttcgtcaaca	tggtaggagca	cgacacgctt	1500
gtctactcca	aaaatatcaa	agatacagtc	tcagaagacc	aaagggcaat	tgagactttt	1560
caacaaaggg	taatatccgg	aaacctcctc	ggattccatt	gcccagctat	ctgtcacttt	1620
attgtgaaga	tagtggaaaa	ggaaggtggc	tcctacaaat	gccatcattg	cgataaagga	1680
aaggccatcg	ttgaagatgc	ctctgccgac	agtggtccca	aagatggacc	cccacccacg	1740
aggagcatcg	tggaaaaaga	agacgttcca	accacgtctt	caaagcaagt	ggattgatgt	1800
gatatactcca	ctgacgtaag	ggatgacgca	caatcccact	atccttcgca	agacccttcc	1860
tctatataag	gaagttcatt	tcatttggag	aggacagggt	accgggggat	ccaccatgtc	1920
tccggagagg	agaccagttg	agattaggcc	agctacagca	gctgatatgg	ccgcggtttg	1980
tgatatcggt	aaccattaca	ttgagacgtc	tacagtgaac	tttaggacag	agccacaaac	2040
accacaagag	tggattgatg	atctagagag	gttgcaagat	agataccctt	ggttggttgc	2100
tgaggttgag	ggtgttgtgg	ctggtattgc	ttacgctggg	ccctggaagg	ctaggaacgc	2160
ttacgattgg	acagttgaga	gtactgttta	cgtgtcacat	aggcatcaaa	ggttgggcct	2220
aggatccaca	ttgtacacac	atttgcttaa	gtctatggag	gcgcaagggt	ttaagtctgt	2280
ggttgctgtt	ataggccttc	caaacgatcc	atctgttagg	ttgcatgagg	ctttgggata	2340
cacagcccg	ggtacattgc	gcgcagctgg	atacaagcat	ggtggatggc	atgatgttgg	2400
tttttgga	agggattttg	agttgccagc	tcctccaagg	ccagttaggc	cagttacca	2460
gatctgagtc	gacctgcagg	catgcccgtc	gaaatcacca	gtctctctct	acaaatctat	2520
ctctctctat	aataatgtgt	gagtagttcc	cagataaggg	aattaggggt	cttatagggt	2580
ttcgtcatg	tgttgagcat	ataagaaacc	cttagtatgt	atttgtat	gtaaaatact	2640
tctatcaata	aaatttctaa	ttcctaaaac	caaaatccag	ggcgagctcg	gtaccggggg	2700
atcctctaga	gtcgacctgc	aggcatgccc	gcggatatcg	atggggcccg	gccgaagctt	2760

US 7,956,246 B2

71

72

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cggtccgggc catcggtggcc tcttgccttt caggatgaag agctatgttt aaacgtgcaa 2820  
gcgctcaatt 2830

<210> SEQ ID NO 28  
<211> LENGTH: 136  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: PCR Amplicon 0784/0564

<400> SEQUENCE: 28

aatccacaag attggagcaa acgaccaaaa attcacaagg attggcggct gacattgcca 60  
gcgcgggatc gcatgcggcg gcggcggcgg gggcgagcac gggagcaggc gacagtcgag 120  
ctccattgga acgtag 136

<210> SEQ ID NO 29  
<211> LENGTH: 263  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: PCR Amplicon 0784/0543

<400> SEQUENCE: 29

aatccacaag attggagcaa acgaccaaaa attcacaagg attggcggct gacattgcca 60  
gcgcgggatc gcatgcggcg gcggcggcgg gggcgagcac gggagcaggc gacagtcgag 120  
ctccattgga acgtagaaat acttaagggc aaggtctcca aatacttgaa aaaataggaa 180  
aaagaagaaa atacatgaaa tgatattgaa atcaattgga agatgttatg aatcttgttt 240  
ttgcaaagcg aacgattcag atg 263

<210> SEQ ID NO 30  
<211> LENGTH: 227  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: PCR Amplicon 0569/0577

<400> SEQUENCE: 30

ggtaagtg acacttggtc actcatttaa tccctccctc tctcttttta tccctctttt 60  
tggtgtattc accaatagtg gtgtgcacct gtgattggct cgtaaaaaatt cttggacgga 120  
tggaagagtg aagagataag caagtcaaaag aaaagtaaca acgaagcttc atcagctaca 180  
aattttggcc caactgggtg caccagcacc aaacttacgt atacatg 227

<210> SEQ ID NO 31  
<211> LENGTH: 492  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: PCR Amplicon 0570/0542

<400> SEQUENCE: 31

gagtgaaag ataagcaagt caaagaaaag taacaacgaa gcttcacag ctacaaattt 60  
tgGCCCAact ggttgcaacca gcaccaaact tacgtataca tgattatctc tgtttccctc 120  
atttcgaaga aaaaaacggg tttcaaaacc cactgctttc aggagtaaaa aaagataata 180  
atctgaaaca ttgcttccac cttggccctt atttggttac gttgcaattc accccaatcc 240  
acatgtggat tgagatggat tgcagtgtag ctagacaaac ccttaggccc tgtttgcata 300  
ggaatacacc aggaattatt ccagctaacc aaaatttata taaatgagag aaacaattcg 360



US 7,956,246 B2

73

74

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gataggaatt gttccaggac ttcattctgc agtaaccgaa cggccccctta atccacccca 420
atacacgtgg attggagtgg attgaggtag agccaaacaa ggcctaagtg cagatcaaat 480
aatcaccccg tc 492

```

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<210> SEQ ID NO 32
<211> LENGTH: 555
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR Amplicon 0784/0215

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<400> SEQUENCE: 32

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aatccacaag attggagcaa acgacaaaaa attcacaagg attggcggct gacattgcca 60
gcgcgggatc gcatgcggcg gcggcggccg gggcgagcac gggagcaggc gacagtcgag 120
ctccattgga acgtagaaat acttaagggc aaggtctcca aatacttgaa aaaataggaa 180
aaagaagaaa atacatgaaa tgatattgaa atcaattgga agatgttatg aatcttgttt 240
ttgcaaagcg aacgattcag atggcaaaac tatgaatctt tttgtttgaa gtcccaata 300
taaaattttc tcgtactcac caacattggt gcgcacctgt gattggctca taaaaattct 360
tgaggggacg gaagaaagag tgaagggata agcaagtaaa agcgctcaaa cactgatagt 420
ttaaactgaa ggcggaagac gacaatctga tcatgagcgg agaattaagg gagtcacgtt 480
atgacccccg ccgatgacgc gggacaagcc gttttacgtt tggaactgac agaaccgcaa 540
cgttgaagga gccac 555

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<210> SEQ ID NO 33
<211> LENGTH: 547
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR Amplicon 0219/0577

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<400> SEQUENCE: 33

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cgtgcaagcg ctcaattcgc cctatagtga gtcgtattac aatcgtaacg aattcagtag 60
attaaaaacg tccgcaatgt gttattaagt tgtctaagcg tcaatttttc ccttctatgg 120
tcccgtttgt ttatctctta aattatataa tccagcttaa ataagttaag agacaaacaa 180
acaacacaga ttattaaata gattatgtaa tctagatacc tagattatgt aatccataag 240
tagaatatca ggtgcttata taatctatga gctcgattat ataactctaa aagaaaacaa 300
acagagcccc tataaaaagg ggtcaagtgg acacttggtc actcatttaa tccctccctc 360
tcctctttta tccctctttt tgggtgtatt accaatagtg gtgtgcacct gtgattggct 420
cgtaaaaatt cttggacgga tggaaagagt aagagataag caagtcaaag aaaagtaaca 480
acgaagcttc atcagctaca aattttggcc caactggttg caccagcacc aaacttacgt 540
atacatg 547

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<210> SEQ ID NO 34
<211> LENGTH: 243
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR Amplicon 0506/0476

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<400> SEQUENCE: 34

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tctcgtaact accaacattg gtgcgcacct gtgattggct cataaaaaatt cttggaggga 60
cggaagaaag agtgaaggga taagcaagta aaagcgctca aacttgata gtttaaactg 120

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US 7,956,246 B2

75

76

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aaggcgggaa acgacaatct gatcatgagc ggagaattaa gggagtcacg ttatgacccc 180
cgccgatgac gcgggacaag ccgttttacg tttggaactg acagaaccgc aacgttgaag 240
gag 243

```

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<210> SEQ ID NO 35
<211> LENGTH: 754
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: PCR Amplicon 0447/0577

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<400> SEQUENCE: 35

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aacccttagt atgtatttgt atttgtaaaa tacttctatc aataaaatct ctaattccta 60
aaacaaaaat ccagggcgag ctcggtaccc ggggatcctc tagagtcgac ctgcaggcat 120
gcccgcggat atcgatgggc cccggccgaa gcttcggtcc gggccatcgt ggccctctgc 180
tcttcaggat gaagagctat gtttaaacgt gcaagcgtc aattcgccct atagtgagtc 240
gtattacaat cgtacgcaat tcagtacatt aaaaacgtcc gcaatgtgtt attaagttgt 300
ctaagcgtca atttttcctt tctatggtcc cgtttggtta tcctctaaat tatataatcc 360
agcttaaata agttaagaga caaacaacaa acacagatta ttaaatagat tatgtaatct 420
agatacctag attatgtaat ccataagtag aatatcaggt gcttatataa tctatgagct 480
cgattatata atcttaaaag aaaacaacaa gagcccctat aaaaaggggt caagtggaca 540
cttggtcact catttaatcc ctccctctcc tcttttatcc ctcttttttg tgtattcacc 600
aatagtgggtg tgcacctgtg attggtctgt aaaaattctt ggacggatgg aagagtgaag 660
agataagcaa gtcaaagaaa agtaacaacg aagcttcac agctacaaat ttggcccaa 720
ctggttgac cagcaccaaa cttacgtata catg 754

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<210> SEQ ID NO 36
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

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<400> SEQUENCE: 36

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cgtattacaa tcgtacgcaa ttcag 25

```

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<210> SEQ ID NO 37
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Oligonucleotide primer

```

```

<400> SEQUENCE: 37

```

```

ggataaacia acgggaccat agaag 25

```

```

<210> SEQ ID NO 38
<211> LENGTH: 104
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Amplicon of SEQ ID NOS: 36 and 37

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<400> SEQUENCE: 38

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cgtattacaa tcgtacgcaa ttcagtacat taaaaacgtc cgcaatgtgt tattaagttg 60
tctaagcgtc aatttttccc ttctatggtc ccgtttgttt atcc 104

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US 7,956,246 B2

77

78

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<210> SEQ ID NO 39  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: IVR1 (0197) Primer used to generate a 226 bp amplicon as an internal positive control

<400> SEQUENCE: 39

ccgctgtatc acaagggtg gtacc

25

<210> SEQ ID NO 40  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: IVR2 (0198) Primer used to generate a 226 bp amplicon as an internal positive control

<400> SEQUENCE: 40

ggagcccggtg tagagcatga cgatc

25

What is claimed is:

1. A corn plant comprising in its genome a DNA construct linked to at least one flanking region, wherein:

- (a) said flanking region comprises a nucleotide sequence selected from the group consisting of the nucleotide sequence set forth in SEQ ID NO: 19 and the nucleotide sequence set forth in SEQ ID NO: 20;
- (b) said DNA construct comprises a first, a second, and a third expression cassette;
- (c) said first expression cassette comprises in operable linkage
  - (i) a maize ubiquitin promoter,
  - (ii) a 5' untranslated exon of a maize ubiquitin gene,
  - (iii) a maize ubiquitin first intron,
  - (iv) a Cry34Abl encoding DNA molecule, and
  - (v) a PinII transcriptional terminator;
- (d) said second expression cassette comprises in operable linkage
  - (vi) a wheat peroxidase promoter,
  - (vii) a Cry35Abl encoding DNA molecule, and
  - (viii) a PinII transcriptional terminator; and
- (e) said third expression cassette comprises in operable linkage
  - (ix) a cauliflower mosaic virus (CaMV) 35S promoter;
  - (x) a pat encoding DNA molecule; and
  - (xi) a 3' transcriptional terminator from CaMV 35S.

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2. The plant of claim 1, wherein said DNA construct comprises the nucleotide sequence set forth in SEQ ID NO: 24.

3. The plant of claim 1, wherein said DNA construct is linked to a first and a second flanking region.

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4. The plant of claim 3, wherein said first flanking region comprises the nucleotide sequence set forth in SEQ ID NO: 19 and said second flanking region comprises the nucleotide sequence set forth in SEQ ID NO: 20.

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5. The plant of claim 4, wherein said DNA construct comprises the nucleotide sequence set forth in SEQ ID NO: 24.

6. The plant of claim 1, wherein said plant is a seed.

7. The plant of claim 2, wherein said plant is a seed.

8. The plant of claim 4, wherein said plant is a seed.

9. The plant of claim 5, wherein said plant is a seed.

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10. A plant comprising in its genome the nucleotide sequence set forth in SEQ ID NO: 23.

11. The plant of claim 10, wherein said plant is a corn plant.

12. A seed comprising in its genome the nucleotide sequence set forth in SEQ ID NO: 23.

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13. The seed of claim 12, wherein said seed is a corn seed.

14. The plant of claim 3, wherein said plant is a seed.

\* \* \* \* \*

# **Exhibit D**

(12) **United States Patent**  
**Wright et al.**

(10) **Patent No.:** **US 8,283,522 B2**  
(45) **Date of Patent:** **Oct. 9, 2012**

(54) **HERBICIDE RESISTANCE GENES**

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(US)

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IN (US)

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patent is extended or adjusted under 35  
U.S.C. 154(b) by 907 days.

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(22) PCT Filed: **Oct. 27, 2006**

(86) PCT No.: **PCT/US2006/042133**

§ 371 (c)(1),

(2), (4) Date: **Nov. 3, 2008**

(87) PCT Pub. No.: **WO2007/053482**

PCT Pub. Date: **May 10, 2007**

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US 2011/0203017 A1 Aug. 18, 2011

**Related U.S. Application Data**

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28, 2005.

(51) **Int. Cl.**  
**C12N 15/82** (2006.01)

(52) **U.S. Cl.** ..... **800/300**; 435/419; 800/298; 800/302

(58) **Field of Classification Search** ..... None  
See application file for complete search history.

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*Primary Examiner* — David H Kruse

(74) *Attorney, Agent, or Firm* — Kenneth B. Ludwig; Jim  
Daly; Faegre Baker Daniels LLP

(57) **ABSTRACT**

The subject invention provides novel plants that are not only  
resistant to 2,4-D, but also to pyridyloxyacetate herbicides.  
Heretofore, there was no expectation or suggestion that a  
plant with both of these advantageous properties could be  
produced by the introduction of a single gene. The subject  
invention also includes plants that produce one or more  
enzymes of the subject invention "stacked" together with one  
or more other herbicide resistance genes. The subject inven-  
tion enables novel combinations of herbicides to be used in  
new ways. Furthermore, the subject invention provides novel  
methods of preventing the development of, and controlling,  
strains of weeds that are resistant to one or more herbicides  
such as glyphosate. The preferred enzyme and gene for use  
according to the subject invention are referred to herein as  
AAD-12 (AryloxyAlkanoate Dioxygenase). This highly  
novel discovery is the basis of significant herbicide tolerant  
crop trait and selectable marker opportunities.

**14 Claims, 3 Drawing Sheets**

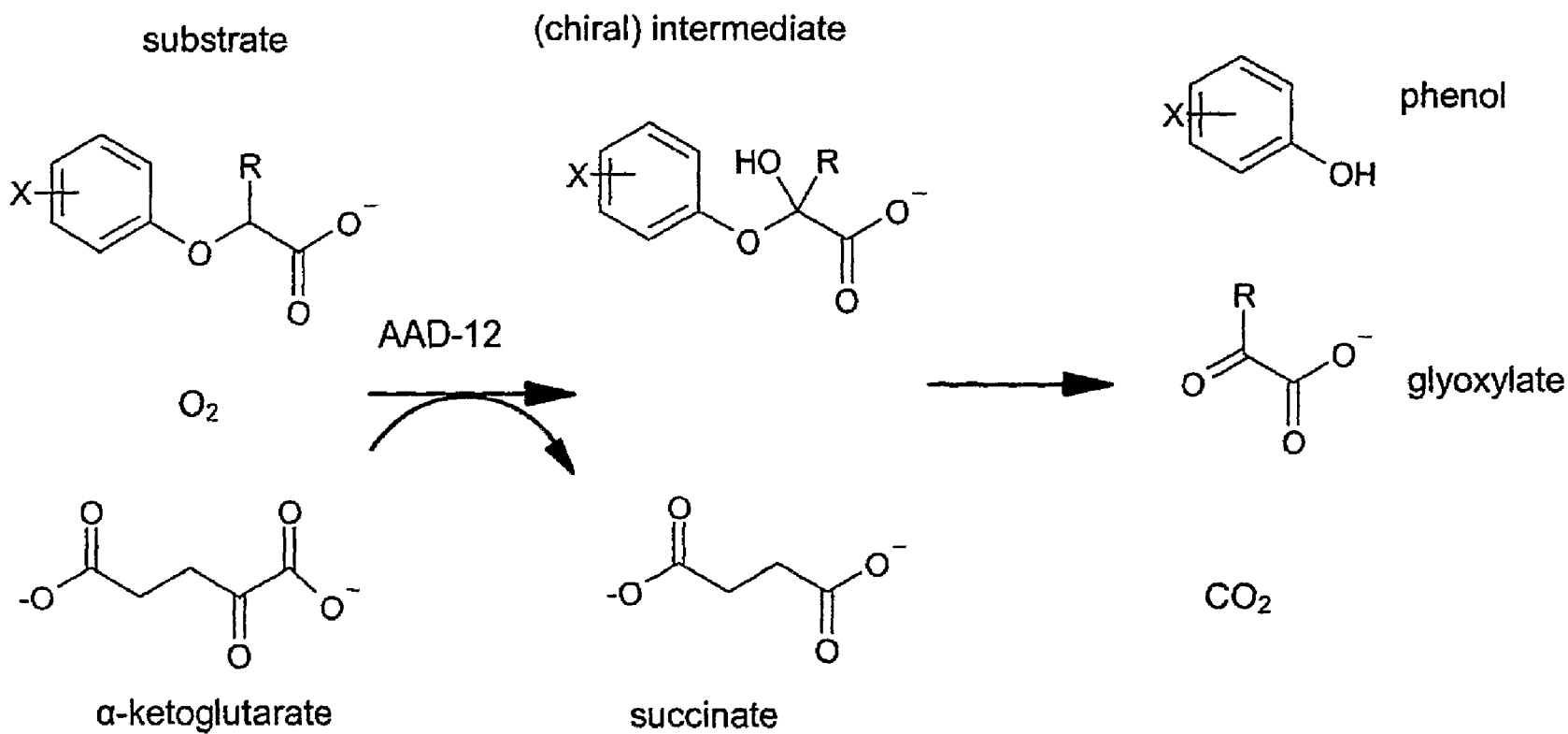


FIG. 1

		10	20	30	40	50	60	70	80	
AAD-12	1	..... ..... ..... ..... ..... ..... ..... ..... ..... .....								
tfdA	1	-----MQTTLQITPTGATIGATVIGVHLAT-LDDAGFAALHAAWLQHALLIFFPGOHLNSNDQOITFAKRFG-----								
AAD-2	1	-----MSVVANPLHPLFAAGVEDIDLREALGSTEVREIERLMDEKSVLVFRGQPLSQDQOIAFARNFGPLEG-----								
AAD-1	1	-----MTIATROLQTHFVGQVSGLDLRKPLTPGEAREVESAMDKYAVLVFHDQDITDEQOMAFALNFGQREDA-----								
tauD	1	-----MHAALSPLSQRFERIAVQPLTGVLCAEITGVDLREPLDDSTWNEILDAFHTYQVIYFPGQAITNEQHIAFSRRFG-----								
		90	100	110	120	130	140	150	160	
AAD-12	64	----AIERIGGGDIVAISNVKADGTVRQHSAPAEWDDMMKVIVGNMAWHAADSTYMPVMAQAVFSAEVVPAVGGRTCFADM								
tfdA	67	-GFIKVNQRPSRFKYAELADISNVSLDGKVAQRDAREVVGNFANQLWHSDSSEFQQPAARYSMLSARVVPPSGGDTFCDM								
AAD-2	69	RGGTVTKEKDYLQ-SGLNDVSNLKGDKPLAKDSRTHLFNLGNCLWHSDSSEFPIPAKFSLLSARVVNPTGGNTEFADM								
AAD-1	75	---PVDPVLLKSIEGYPEVQIMIRREANESGR-----VIGDDWHTDSTFLDAPPAAVVMRAIDVPEHGGDTGFLSM								
tauD	65	----ELHIHVPYPHAEGVDEIIVLDTHNDNPP-----DNDNWHIDVTETETPPAGAILAAKELPSTGGDTLWTS								
		***** ** *								
		170	180	190	200	210	220	230	240	
AAD-12	141	RAAYDALDEATRALVHQRSARHSLVYSQS---KLGHVQOAGSAVIGYGMDDTATPLRPLVKVHPETGRPSLLIGR-HAH								
tfdA	147	RAAYDALPRDLQSELEGLRAEHYALNSRF-----LLGDDTYSEAQRNAMPPVNWPLVRTHAGSGRKFLFIGA-HAS								
AAD-2	148	RAAYDALDDETKAEIEDLVCEHSLMYSRG-----SLGFTEYTDEEKQMFKEVLQRLVTRHPVHRRKSLYLSS-HAG								
AAD-1	144	YTAWETLSPTMQATTIEGLNVVHSATRVFGSLYQAQNRFRSNTSVKVMVDVDAGDRETVEPLVTHPGSGRKGLYVNQVYCQ								
tauD	132	IAAYBALSVPPFRQLLSGLRAEHDFRKSFP---EYKYRKTEEBHQRWREAVAKNPPELLHPVVRTHPVSKQALFVNEGFTT								
		***** ** * * *								
		250	260	270	280	290	300	310		
AAD-12	216	AIPGMDAAESERFLEGLVDWACQAPRVHAHQWAAGDVVVWDNRCLLHRAEPWDFKLPVVMWHSRLAGRPETEGAALV-								
tfdA	217	HVEGLPVAEGRMLLAELLEHATQREFVYRHRWNVGDVLMWDNRCLVLRGRRYDISARRELNRATFLDDAVV-----								
AAD-2	218	KIASMSVPEGRLLLRDINEHATQPEFVYVHKWKLHDLVMWDNRQTMHRVRRYDQSOPRDMRRATVAGTEPTVQQQAAE								
AAD-1	224	RIEGMTDAESKPLLQFLYEHAIRFDFTCRVRWKKDQVLVWDNLCTMHRVAVPDYAGKFRYLTRITVGCVRPAR-----								
tauD	209	RIVDVSEKESEALLSELEFAHITKPEFQVRWRWQPNDAIWDNRVTOHYANADYLPQRRIMRATILGDKPFYRAG---								
		* * ***** *								

FIG. 2



U.S. Patent

Oct. 9, 2012

Sheet 3 of 3

US 8,283,522 B2

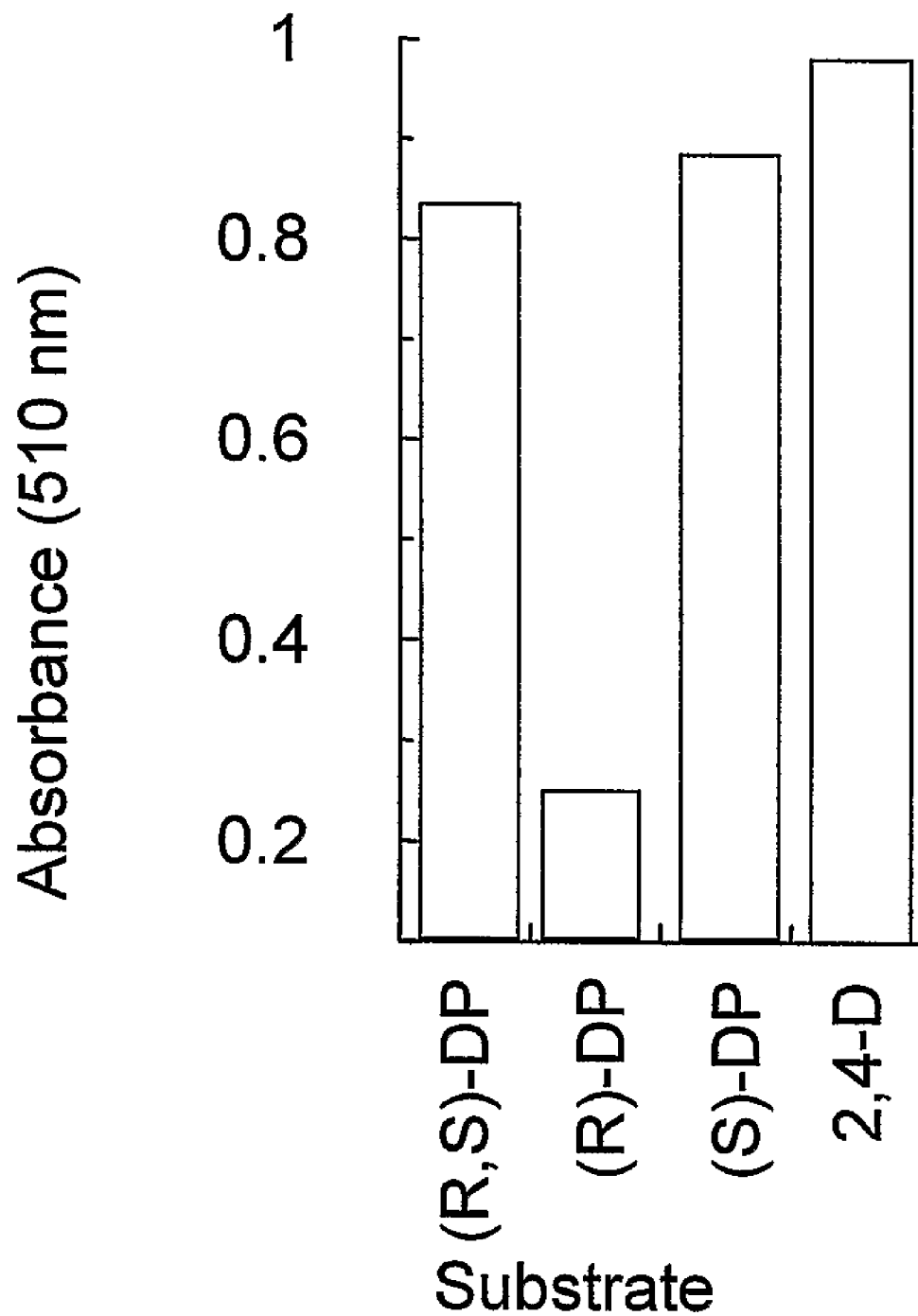


FIG. 3

## US 8,283,522 B2

1

## HERBICIDE RESISTANCE GENES

## CROSS-REFERENCE TO RELATED APPLICATION

This application is the U.S. national stage application of International Patent Application No. PCT/US2006/042133, filed Oct. 27, 2006, which claims the benefit of U.S. Provisional Patent Application No. 60/731,044, filed Oct. 28, 2005, the disclosures of which are hereby incorporated by reference in their entireties, including all figures, tables and amino acid or nucleic acid sequences.

## BACKGROUND OF THE INVENTION

Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant soybeans, for example, can be sprayed to control weeds without significantly damaging the soybean plants.

With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. Alfalfa was the first perennial GTC introduced, furthering the opportunity for repeated use of glyphosate on the same crop and fields repeatedly over a period of years. More GTCs (e.g., wheat, rice, sugar beets, turf, etc.) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (e.g., sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; see "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (i.e., weed shifts). (Powles and Preston, 2006; Ng et al., 2003; Simarmata et al., 2003; Lorraine-Colwill et al., 2003; Sfiligoj, 2004; Miller et al., 2003; Heap, 2005; Murphy et al., 2002; Martin et al., 2002.) Although glyphosate has been widely

2

used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past five years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum*, *Lolium multiflorum*, *Eleusine indica*, *Sorghum halepense*, *Ambrosia artemisiifolia*, *Conyza canadensis*, *Conyza bonariensis*, *Plantago lanceolata*, *Amaranthus palmerii*, and *Amaranthus rudis*. Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea*, *Amaranthus*, *Chenopodium*, *Taraxacum*, and *Commelina species*.

In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agrilience, 2005).

2,4-D is in the phenoxy acid class of herbicides, as is MCPA. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known. Triclopyr and fluoroxyppr are pyridyloxyacetic acid herbicides whose mode of action is as a synthetic auxin, also.

These herbicides have different levels of selectivity on certain plants (e.g., dicots are more sensitive than grasses). Differential metabolism by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D currently formulated has a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

US 8,283,522 B2

3

One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an  $\alpha$ -ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al. (1989), Lyon et al. (1989), Lyon (1993), and U.S. Pat. No. 5,608,147).

A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with  $\alpha$ -ketoglutarate dioxygenase  $\text{Fe}^{+2}$  dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

One unique example with low homology to *tfdA* (31% amino acid identity) is *sdpA* from *Delftia acidovorans* (Kohler et al., 1999, Westendorf et al., 2002, Westendorf et al., 2003). This enzyme has been shown to catalyze the first step in (S)-dichlorprop (and other (S)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf et al., 2003). Transformation of this gene into plants, has not heretofore been reported.

Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxyacetate auxins (such as 2,4-D and dichlorprop), pyridyloxyacetate auxins (such as fluoroxyppyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. A multifunctional enzyme for the degradation of herbicides covering multiple modes of action has recently been described (PCT US/2005/014737; filed May 2, 2005). Another unique multifunctional enzyme and potential uses are described hereafter.

#### BRIEF SUMMARY OF THE INVENTION

The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, ALS- (imidazolinone, sulfonyleurea), aryloxyalkanoate-, HPPD-, PPO-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide

4

resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, MCPA, triclopyr, fluoroxyppyr, and one or more commercially available herbicides (e.g., glyphosate, glufosinate, paraquat, ALS-inhibitors (e.g., sulfonyleureas, imidazolinones, triazolopyrimidine sulfonanilides, et al), HPPD inhibitors (e.g. mesotrione, isoxaflutole, et al.), dicamba, bromoxynil, aryloxyphenoxypropionates, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including preplant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and pyridyloxyacetate herbicides. No  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of both the phenoxyacetate and pyridyloxyacetates auxin herbicides. The preferred enzyme and gene for use according to the subject invention are referred to herein as AAD-12 (Aryloxyalkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide-tolerant crop (HTC) trait and selectable marker opportunities. Plants of the subject invention can be resistant throughout their entire life cycle.

There was no prior motivation to produce plants comprising an AAD-12 gene (preferably an AAD-12 polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-12 enzyme to render the plants resistant a phenoxyacetic acid herbicide (such as 2,4-D) and/or one or more pyridyloxyacetates herbicides such as triclopyr and fluoroxyppyr. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxyacetate auxin and/or pyridyloxyacetates auxin herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid and other aryloxyalkanoate auxin herbicides by a recombinantly expressed AAD-12 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more pyridyloxyacetate or phenoxyacetate auxin herbicides to plants comprising an AAD-12 gene. The subject invention also provides methods of using an AAD-12 gene as a selectable marker for identifying plant cells and whole plants transformed with AAD-12, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention

## US 8,283,522 B2

5

include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

## BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates the general chemical reaction that is catalyzed by AAD-12 enzymes of the subject invention.

FIG. 2 is an amino acid sequence alignment of an exemplified AAD-12 protein, TfdA, AAD-2, AAD-1, and TauD.

FIG. 3 illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop.

## BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the nucleotide sequence of AAD-12 from *Delftia acidovorans*.

SEQ ID NO:2 is the translated protein sequence encoded by SEQ ID NO:1.

SEQ ID NO:3 is the plant optimized nucleotide sequence of AAD-12 (v1).

SEQ ID NO:4 is the translated protein sequence encoded by SEQ ID NO:3.

SEQ ID NO:5 is the *E. coli* optimized nucleotide sequence of AAD-12 (v2).

SEQ ID NO:6 is the sequence of the M13 forward primer.

SEQ ID NO:7 is the sequence of the M13 reverse primer.

SEQ ID NO:8 is the sequence of the forward AAD-12 (v1) PTU primer.

SEQ ID NO:9 is the sequence of the reverse AAD-12 (v1) PTU primer.

SEQ ID NO:10 is the sequence of the forward AAD-12 (v1) coding PCR primer.

SEQ ID NO:11 is the sequence of the reverse AAD-12 (v1) coding PCR primer.

SEQ ID NO:12 shows the sequence of the "sdpacodF" AAD-12 (v1) primer.

SEQ ID NO:13 shows the sequence of the "sdpacodR" AAD-12 (v1) primer.

SEQ ID NO:14 shows the sequence of the "Nco1 of Brady" primer.

SEQ ID NO:15 shows the sequence of the "Sac1 of Brady" primer.

6

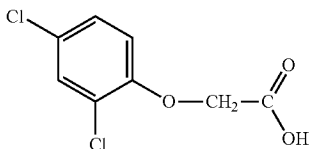
SEQ ID NO:16 provides the sequence of forward primer brjap 5'(spel).

SEQ ID NO:17 provides the sequence of reverse primer brjap 3'(xhol).

## DETAILED DESCRIPTION OF THE INVENTION

The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of the subject herbicide tolerance trait for 2,4-D is its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism, and the like. An additional benefit of the AAD-12 gene is that unlike all tfdA homologues characterized to date, AAD-12 is able to degrade the pyridyloxyacetates auxins (e.g., triclopyr, fluoroxypry) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid). See Table 1. A general illustration of the chemical reactions catalyzed by the subject AAD-12 enzyme is shown in FIG. 1. (Addition of O<sub>2</sub> is stereospecific; breakdown of intermediate to phenol and glyoxylate is spontaneous.) It should be understood that the chemical structures in FIG. 1 illustrate the molecular backbones and that various R groups and the like (such as those shown in Table 1) are included but are not necessarily specifically illustrated in FIG. 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the AAD-12 gene in plants affords protection to a much wider spectrum of auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled. The subject invention can also be used to protect from drift or other off-site synthetic auxin herbicide injury for the full breadth of commercially available phenoxy auxins. Table 1 defines commercially available pyridyloxy and phenoxy auxins and provides relevant chemical structures.

TABLE 1

Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme in planta as general plant esterases convert these esters to the active acids in planta. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.				
Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	

US 8,283,522 B2

7

8

TABLE 1-continued

Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme in planta as general plant esterases convert these esters to the active acids in planta. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4,5-T	93-76-5	25-4000	25-4000	
4-CPA	122-88-3	25-4000	25-4000	
3,4-DA	588-22-7	25-4000	25-4000	
MCPA	94-74-6	25-4000	125-1550	
Triclopyr	55335-06-3	50-2000	70-840	
Fluroxypyr	69377-81-7	25-2000	35-560	

A single gene (AAD-12) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Additionally, AAD-12 can provide protection in planta to pyridyloxyacetate herbicides where natural tolerance also was not sufficient to allow selectivity, expanding the potential utility of these herbicides. Plants containing AAD-12 alone now may be treated sequentially or tank mixed with one, two, or a combination of several phenoxy auxin herbicides. The rate

for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several pyridyloxyacetate auxin compounds may be applied to plants expressing AAD-12 with reduced risk of injury from said herbicides. The rate for each pyridyloxyacetate herbicide may range from 25 to 2000 g ae/ha, and more typically from 35-840 g ae/ha for the control of additional dicot weeds.

Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However,



US 8,283,522 B2

9

repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking AAD-12 with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant dicot weed species in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (e.g., 2,4-D) and pyridyloxyacetates auxin herbicides (e.g., triclopyr)—selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from approximately 2 hours to approximately 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within about 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-12 stack could range from about 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from about 25-4000 g ae/ha; and pyridyloxyacetates auxin herbicide(s) (one or more) could be applied from 25-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

Plantlets are typically resistant throughout the entire growing cycle. Transformed plants will typically be resistant to new herbicide application at any time the gene is expressed. Tolerance is shown herein to 2,4-D across the life cycle using the constitutive promoters tested thus far (primarily CsVMV and AtUbi 10). One would typically expect this, but it is an improvement upon other non-metabolic activities where tolerance can be significantly impacted by the reduced expression of a site of action mechanism of resistance, for example. One example is Roundup Ready cotton, where the plants were tolerant if sprayed early, but if sprayed too late the glyphosate concentrated in the meristems (because it is not metabolized and is translocated); viral promoters Monsanto used are not well expressed in the flowers. The subject invention provides an improvement in these regards.

Herbicide formulations (e.g., ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (e.g., adjuvants, surfactants, drift retardants, or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant or resistant weed species. This could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or

10

non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (e.g., resistant plant or bacterial EPSPS, glyphosate oxidoreductase (GOX), GAT), glufosinate resistance (e.g., Pat, bar), acetolactate synthase (ALS)-inhibiting herbicide resistance (e.g., imidazolinone, sulfonyleurea, triazolopyrimidine sulfonanilide, pyrimidinylthiobenzoates, and other chemistries=AHAS, Csr1, SurA, et al.), bromoxynil resistance (e.g., Bxn), resistance to inhibitors of HPPD (4-hydroxyphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (e.g., psbA), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (e.g., PPO-1), resistance to phenylurea herbicides (e.g., CYP76B1), dicamba-degrading enzymes (see, e.g., US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes. In vivo modified EPSPS can be used in some preferred embodiments, as well as Class I, Class II, and Class III glyphosate resistance genes.

Regarding additional herbicides, some additional preferred ALS inhibitors include but are not limited to the sulfonyleureas (such as chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron), imidazolinones (such as imazamox, imazethapyr, imazaquin), triazolopyrimidine sulfonanilides (such as clorasulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyriothiac), and flucarbazone. Some preferred HPPD inhibitors include but are not limited to mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include but are not limited to flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

Additionally, AAD-12 alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known ttdA proteins, for example. Even though this enzyme has very low homology to ttdA, the genes of the subject invention can still be generally classified in the same overall family of  $\alpha$ -ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "HX(D/E)X<sub>23-26</sub>(T/S)X<sub>114-183</sub>HX<sub>10-13</sub>R" motif which comprises the active site. The histidines coordinate Fe<sup>+2</sup> ion in the active site that is essential for catalytic activity (Hogan et al., 2000). The preliminary in vitro expression experiments discussed herein were tailored to help select for novel attributes. These experiments also indicate the AAD-12 enzyme is unique from another disparate enzyme of the same class, disclosed in a previously filed patent application (PCT US/2005/014737; filed May 2, 2005). The AAD-12 enzyme of that application shares only about 25% sequence identity with the subject AAD-12 protein.

More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D,

## US 8,283,522 B2

11

but also pyridyloxyacetate herbicides. No  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase) genes and proteins.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and pyridyloxyacetate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic acids, and pyridyloxyacetic acid herbicides by a recombinantly expressed AAD-12 enzyme.

The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP in vitro. An additional advantage that AAD-12 transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

The subject invention also includes methods of controlling weeds wherein said methods comprise applying a pyridyloxyacetate and/or a phenoxy auxin herbicide to plants comprising an AAD-12 gene.

In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also pyridyloxyacetate herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an  $\alpha$ -ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al., 1989; Lyon et al., 1989; Lyon et al., 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of  $\alpha$ -ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

The subject invention relates in part to surprising discoveries of new uses for and functions of a distantly related enzyme, *sdpA*, from *Delftia acidivorans* (Westendorf et al., 2002, 2003) with low homology to *tfdA* (31% amino acid identity). This  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and S-dichloroprop (Westendorf et al., 2002

12

and 2003). However, no  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of pyridyloxyacetate chemical class. *SdpA* has never been expressed in plants, nor was there any motivation to do so in part because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-12 proteins and genes. AAD-12 was presently confirmed to degrade a variety of phenoxyacetate auxin herbicides in vitro. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance include the pyridyloxyacetate auxin herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxyacetate and pyridyloxyacetate auxins).

Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic acid herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-12) capable of degrading phenoxy and/or pyridyloxy auxin herbicides.

The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf weeds. AAD-12 can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits [e.g., glyphosate resistance, glufosinate resistance, ALS-inhibitor (e.g., imidazolinone, sulfonylurea, triazopyrimidine sulfonamide) resistance, bromoxynil resistance, HPPD-inhibitor resistance, PPO-inhibitor resistance, et al.], and insect resistance traits (Cry1F, Cry1Ab, Cry 34/45, other Bt. Proteins, or insecticidal proteins of a non-*Bacillus* origin, et al.) for example. Additionally, AAD-12 can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Ara-bidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with AAD-12-containing constructs and have demonstrated high levels of resistance to both the phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to "plant optimized" genes that encode proteins of the subject invention.

Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by "acid trapping," a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-12. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to



## US 8,283,522 B2

13

herbicides (e.g., glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

The transgenic expression of the subject AAD-12 genes is exemplified in, for example, *Arabidopsis*, tobacco, soybean, cotton, rice, corn and canola. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be utilized in multiple other monocot (such as pasture grasses or turf grass) and dicot crops like alfalfa, clover, tree species, et al. Likewise, 2,4-D (or other AAD-12-substrates) can be more positively utilized in grass crops where tolerance is moderate, and increased tolerance via this trait would provide growers the opportunity to use these herbicides at more efficacious rates and over a wider application timing without the risk of crop injury.

Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed. This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-12 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-12 for other aryloxyalkanoate auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

Proteins (and source isolates) of the subject invention. The present invention provides functional proteins. By “functional activity” (or “active”) it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce “an effective amount” of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of

14

effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

One cannot easily discuss the term “resistance” and not use the verb “tolerate” or the adjective “tolerant.” The industry has spent innumerable hours debating Herbicide Tolerant Crops (HTC) versus Herbicide Resistant Crops (HRC). HTC is a preferred term in the industry. However, the official Weed Science Society of America definition of resistance is “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis.” As used herein unless otherwise indicated, herbicide “resistance” is heritable and allows a plant to grow and reproduce in the presence of a typical herbicidally effective treatment by a herbicide for a given plant, as suggested by the current edition of *The Herbicide Handbook* as of the filing of the subject disclosure. As is recognized by those skilled in the art, a plant may still be considered “resistant” even though some degree of plant injury from herbicidal exposure is apparent. As used herein, the term “tolerance” is broader than the term “resistance,” and includes “resistance” as defined herein, as well as an improved capacity of a particular plant to withstand the various degrees of herbicidally induced injury that typically result in wild-type plants of the same genotype at the same herbicidal dose.

Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein’s amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

US 8,283,522 B2

15

One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria.

Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutant strains can also be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook et al., 1989, which is incorporated herein by reference.

Polynucleotides and probes. The subject invention further provides nucleic acid sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the AAD-12 gene constitutively expressed in all tissues for complete protection of the plant at all growth-stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage. In addition, desired levels and times of expression can also depend on the type of plant and the level of tolerance desired. Some preferred

16

embodiments use strong constitutive promoters combined with transcription enhancers and the like to increase expression levels and to enhance tolerance to desired levels. Some such applications are discussed in more detail below, before the Examples section.

As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein *in vivo*, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, etc.).

As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G. H., M. M. Manak (1987) *DNA Probes*, Stockton Press, New York, N.Y., pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2xSSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for

## US 8,283,522 B2

17

15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1×SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1×SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2×SSC/0.1% SDS can be prepared by adding 50 ml of 20×SSC and 5 ml of 10% SDS to 445 ml of water. 20×SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

As used herein, “stringent” conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with <sup>32</sup>P-labeled gene-specific probes can be performed by standard methods (see, e.g., Maniatis et al. 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C. below the melting temperature (T<sub>m</sub>) of the DNA hybrid in 6×SSPE, 5×Denhardt’s solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz et al. 1983):

$$T_m = 81.5^\circ \text{C.} + 16.6 \log [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.61 (\% \text{ formamide}) - 600 / \text{length of duplex in base pairs.}$$

Washes can typically be carried out as follows:

- (1) Twice at room temperature for 15 minutes in 1×SSPE, 0.1% SDS (low stringency wash).
- (2) Once at T<sub>m</sub>-20° C. for 15 minutes in 0.2×SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C. below the melting temperature (T<sub>m</sub>) of the hybrid in 6×SSPE, 5×Denhardt’s solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T<sub>m</sub> for oligonucleotide probes can be determined by the following formula:

$$T_m (^\circ \text{C.}) = 2(\text{number T/A base pairs}) + 4(\text{number G/C base pairs})$$

(Suggs et al., 1981).

Washes can typically be out as follows:

- (1) Twice at room temperature for 15 minutes 1×SSPE, 0.1% SDS (low stringency wash).

18

- (2) Once at the hybridization temperature for 15 minutes in 1×SSPE, 0.1% SDS (moderate stringency wash).

In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

Low: 1 or 2×SSPE, room temperature

Low: 1 or 2×SSPE, 42° C.

Moderate: 0.2× or 1×SSPE, 65° C.

High: 0.1×SSPE, 65° C.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

PCR technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see Mullis, U.S. Pat. Nos. 4,683, 195, 4,683,202, and 4,800,159; Saiki et al., 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Tag polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. “Variant” genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein.



## US 8,283,522 B2

19

The top two results of BLAST searches with the native aad-12 nucleotide sequence show a reasonable level of homology (about 85%) over 120 basepairs of sequence. Hybridization under certain conditions could be expected to include these two sequences. See GENBANK Acc. Nos. DQ406818.1 (89329742; *Rhodoferrax*) and AJ6288601.1 (44903451; *Sphingomonas*). *Rhodoferrax* is very similar to *Delftia* but *Sphingomonas* is an entirely different Class phylogenetically.

The terms “variant proteins” and “equivalent proteins” refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein, reference to an “equivalent” sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

In addition, U.S. Pat. No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene “shuffling,” which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

“Shuffling” can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, “focused shuffling” can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

Specific changes to the “active site” of the enzyme can be made to affect the inherent functionality with respect to activity or stereospecificity (see alignment FIG. 2). Muller et al. (2006). The known tauD crystal structure was used as a model dioxygenase to determine active site residues while bound to its inherent substrate taurine. Elkins et al. (2002) “X-ray crystal structure of *Escherichia coli* taurine/alpha-ketoglutarate dioxygenase complexed to ferrous iron and substrates,” *Biochemistry* 41(16):5185-5192. Regarding sequence optimization and designability of enzyme active sites, see Chakrabarti et al., PNAS, (Aug. 23, 2005), 102(34): 12035-12040.

Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these “gene shuffling” techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10,

20

11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, or 293 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By “truncated protein” it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, B.t. proteins can be used in a truncated (core protein) form (see, e.g., Höfte et al. (1989), and Adang et al. (1985)). As used herein, the term “protein” can include functionally active truncations.

In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant

or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990. BLAST nucleotide searches are performed with the NBLAST program, score=100, wordlength=12. Gapped BLAST can be used as described in Altschul et al., 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. See NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, Md., U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. Table 2 provides a listing of examples of amino acids belonging to each class.

TABLE 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an

"isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

Optimization of sequence for expression in plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

Transgenic hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf, legume forages (e.g., alfalfa and clover), pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

Although plants can be preferred, the subject invention also includes production of highly active recombinant AAD-12 in a *Pseudomonas fluorescens* (Pf) host strain, for example. The subject invention includes preferred growth temperatures for maintaining soluble active AAD-12 in this host; a fermentation condition where AAD-12 is produced as more than 40% total cell protein, or at least 10 g/L; a purification process results high recovery of active recombinant AAD-12 from a Pf host; a purification scheme which yields at least 10 g active AAD-12 per kg of cells; a purification scheme which can

## US 8,283,522 B2

23

yield 20 g active AAD-12 per kg of cells; a formulation process that can store and restore AAD-12 activity in solution; and a lyophilization process that can retain AAD-12 activity for long-term storage and shelf life.

Insertion of genes to form transgenic hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Pat. No. 5,135,867.

Vectors comprising an AAD-12 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13 mp series, pACYC184, etc. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley et al. (1986); and An et al. (1985).

A large number of techniques are available for inserting DNA into a plant host cell.

Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Hol-

24

sters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, inter alia. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate (e.g., PAT/bar), glyphosate (EPSPS), ALS-inhibitors (e.g., imidazolinone, sulfonylurea, triazopyrimidine sulfonanilide, et al.), bromoxynil, HPPD-inhibitor resistance, PPO-inhibitors, ACC-ase inhibitors, and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. Nos. 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow Agro-Sciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, see U.S. Pat. Nos. 5,177,010 to University of Toledo; 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Pat. Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Pat. No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Pat. No. 5,231,019, all to



## US 8,283,522 B2

25

Ciba Geigy, now Syngenta; U.S. Pat. Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Pat. Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. See U.S. Pat. Nos. 5,302, 523 and 5,464,765, both to Zeneca, now Syngenta. Other direct DNA delivery transformation technology includes aerosol beam technology. See U.S. Pat. No. 6,809,232. Electroporation technology has also been used to transform plants. See WO 87/06614 to Boyce Thompson Institute; U.S. Pat. Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Pat. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al. (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish

26

future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G41; hygromycin resistance; methotrexate resistance, as well as those genes which encode for resistance or tolerance to glyphosate; phosphinothricin (bialaphos or glufosinate); ALS-inhibiting herbicides (imidazolinones, sulfonylureas and triazolopyrimidine herbicides), ACC-ase inhibitors (e.g., aryloxypropionates or cyclohexanediones), and others such as bromoxynil, and HPPD-inhibitors (e.g., mesotrione) and the like.

In addition to a selectable marker, it may be desirable to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising et al., 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the uidA locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the uidA locus of *E. coli* as described by Jefferson et al., (1987) to identify transformed cells.

In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, see U.S. Pat. No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expres-



sion or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Pat. No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Pat. Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

Means of further increasing tolerance or resistance levels. It is shown herein that plants of the subject invention can be imparted with novel herbicide resistance traits without observable adverse effects on phenotype including yield. Such plants are within the scope of the subject invention. Plants exemplified and suggested herein can withstand 2x, 3x, 4x, and 5x typical application levels, for example, of at least one subject herbicide. Improvements in these tolerance levels are within the scope of this invention. For example, various techniques are known in the art, and can foreseeably be optimized and further developed, for increasing expression of a given gene.

One such method includes increasing the copy number of the subject AAD-12 genes (in expression cassettes and the like). Transformation events can also be selected for those having multiple copies of the genes.

Strong promoters and enhancers can be used to "supercharge" expression. Examples of such promoters include the preferred 35T promoter which uses 35S enhancers. 35S, maize ubiquitin, *Arabidopsis* ubiquitin, A.t. actin, and CSMV promoters are included for such uses. Other strong viral promoters are also preferred. Enhancers include 4 OCS and the 35S double enhancer. Matrix attachment regions (MARs) can also be used to increase transformation efficiencies and transgene expression, for example.

Shuffling (directed evolution) and transcription factors can also be used for embodiments according to the subject invention.

Variant proteins can also be designed that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like. See e.g. U.S. Pat. No. 7,058,515; Larson et al., *Protein Sci.* 2002 11: 2804-2813, "Thoroughly sampling sequence space: Large-scale protein design of structural ensembles."; Cramer et al., *Nature Biotechnology* 15, 436-438 (1997), "Molecular evolution of an arsenate detoxification pathway by DNA shuffling."; Stemmer, W. P. C. 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. *Proc. Natl. Acad. Sci. USA* 91: 10747-10751; Stemmer, W. P. C. 1994. Rapid evolution of a protein in vitro by DNA shuffling. *Nature* 370: 389-391; Stemmer, W. P. C. 1995. Searching sequence space. *Bio/Technology* 13: 549-553; Cramer, A., Swirls, S. and Stemmer, W. P. C. 1996. Construction and evolution of antibody-phage libraries by DNA shuffling. *Nature Medicine* 2: 100-103; and Cramer, A., Whitehorn, E. A., Tate, E. and Stemmer, W. P. C. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnology* 14: 315-319.

The activity of recombinant polynucleotides inserted into plant cells can be dependent upon the influence of endogenous plant DNA adjacent the insert. Thus, another option is taking advantage of events that are known to be excellent locations in a plant genome for insertions. See e.g. WO 2005/103266 A1, relating to cry1F and cry1Ac cotton events; the subject AAD-12 gene can be substituted in those genomic loci in place of the cry1F and/or cry1Ac inserts. Thus, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (USPA 20030232410), relating to the use of zinc fingers for targeted recombination. The use of recombinases (cre-10x and flip-frt for example) is also known in the art.

AAD-12 detoxification is believed to occur in the cytoplasm. Thus, means for further stabilizing this protein and mRNAs (including blocking mRNA degradation) are included in aspects of the subject invention, and art-known techniques can be applied accordingly. The subject proteins can be designed to resist degradation by proteases and the like (protease cleavage sites can be effectively removed by re-engineering the amino acid sequence of the protein). Such embodiments include the use of 5' and 3' stem loop structures like UTRs from osmotin, and per5 (AU-rich untranslated 5' sequences). 5' caps like 7-methyl or 2'-O-methyl groups, e.g., 7-methylguanylic acid residue, can also be used. See, e.g.: *Proc. Natl. Acad. Sci. USA* Vol. 74, No. 7, pp. 2734-2738 (July 1977) *Importance of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis*. Protein complexes or ligand blocking groups can also be used.

Computational design of 5' or 3' UTR most suitable for AAD-12 (synthetic hairpins) can also be conducted within the scope of the subject invention. Computer modeling in general, as well as gene shuffling and directed evolution, are discussed elsewhere herein. More specifically regarding computer modeling and UTRs, computer modeling techniques for use in predicting/evaluating 5' and 3' UTR derivatives of the present invention include, but are not limited to: MFold version 3.1 available from Genetics Corporation Group, Madison, Wis. (see Zucker et al., *Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide*. In *RNA Biochemistry and Biotechnology*, 11-43, J. Barciszewski & B. F. C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, Dordrecht, NL, (1999); Zucker et al., *Expanded Sequence Dependence of Thermody-*

*namic Parameters Improves Prediction of RNA Secondary Structure.* *J. Mol. Biol.* 288, 911-940 (1999); Zucker et al., RNA Secondary Structure Prediction. In *Current Protocols in Nucleic Acid Chemistry* S. Beaucage, D. E. Bergstrom, G. D. Glick, and R. A. Jones eds., John Wiley & Sons, New York, 11.2.1-11.2.10, (2000)), COVE (RNA structure analysis using covariance models (stochastic context free grammar methods)) v. 2.4.2 (Eddy & Durbin, *Nucl. Acids Res.* 1994, 22: 2079-2088) which is freely distributed as source code and which can be downloaded by accessing the website [genetics.wustl.edu/eddy/software/](http://genetics.wustl.edu/eddy/software/), and FOLDALIGN, also freely distributed and available for downloading at the website [bioinf.au.dk.FOLDALIGN/](http://bioinf.au.dk/FOLDALIGN/) (see *Finding the most significant common sequence and structure motifs in a set of RNA sequences.* J. Gorodkin, L. J. Heyer and G. D. Stormo. *Nucleic Acids Research*, Vol. 25, no. 18 pp 3724-3732, 1997; *Finding Common Sequence and Structure Motifs in a set of RNA Sequences.* J. Gorodkin, L. J. Heyer, and G. D. Stormo. *ISMB* 5;120-123, 1997).

Embodiments of the subject invention can be used in conjunction with naturally evolved or chemically induced mutants (mutants can be selected by screening techniques, then transformed with AAD-12 and possibly other genes). Plants of the subject invention can be combined with ALS resistance and/or evolved glyphosate resistance. Aminopyralid resistance, for example, can also be combined or "stacked" with an AAD-12 gene.

Traditional breeding techniques can also be combined with the subject invention to powerfully combine, introgress, and improve desired traits.

Further improvements also include use with appropriate safeners to further protect plants and/or to add cross resistance to more herbicides. (Safeners typically act to increase plants immune system by activating/expressing cP450. Safeners are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism, without compromising weed control efficacy.)

Herbicide safeners include benoxacor, cloquintocet, cyometrinil, dichlormid, dicyclonon, dietholate, fenchlorazole, fenclorim, flurazole, fluxofenim, furilazole, isoxadifen, mefenpyr, mephenate, naphthalic anhydride, and oxabetrinil. Plant activators (a new class of compounds that protect plants by activating their defense mechanisms) can also be used in embodiments of the subject invention. These include acibenzolar and probenazole.

Commercialized safeners can be used for the protection of large-seeded grass crops, such as corn, grain sorghum, and wet-sown rice, against preplant-incorporated or preemergence-applied herbicides of the thiocarbamate and chloroacetanilide families. Safeners also have been developed to protect winter cereal crops such as wheat against postemergence applications of aryloxyphenoxypionate and sulfonylurea herbicides. The use of safeners for the protection of corn and rice against sulfonylurea, imidazolinone, cyclohexanedione, isoxazole, and triketone herbicides is also well-established. A safener-induced enhancement of herbicide detoxification in safened plants is widely accepted as the major mechanism involved in safener action. Safeners induce cofactors such as glutathione and herbicide-detoxifying enzymes such as glutathione S-transferases, cytochrome P450 monooxygenases, and glucosyl transferases. Hatzios K K, Burgos N (2004) "Metabolism-based herbicide resistance: regulation by safeners," *Weed Science*: Vol. 52, No. 3 pp. 454-467.

Use of a cytochrome p450 monooxygenase gene stacked with AAD-12 is one preferred embodiment. There are P450s involved in herbicide metabolism; cP450 can be of mammalian or plant origin, for example. In higher plants, cytochrome

P450 monooxygenase (P450) is known to conduct secondary metabolism. It also plays an important role in the oxidative metabolism of xenobiotics in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase). Resistance to some herbicides has been reported as a result of the metabolism by P450 as well as glutathione S-transferase. A number of microsomal P450 species involved in xenobiotic metabolism in mammals have been characterized by molecular cloning. Some of them were reported to metabolize several herbicides efficiently. Thus, transgenic plants with plant or mammalian P450 can show resistance to several herbicides.

One preferred embodiment of the foregoing is the use of cP450 for resistance to acetochlor (acetochlor-based products include Surpass®, Keystone®, Keystone LA, FulTime® and TopNotch® herbicides) and/or trifluralin (such as Treflan®). Such resistance in soybeans and/or corn is included in some preferred embodiments. For additional guidance regarding such embodiments, see e.g. Inui et al., "A selectable marker using cytochrome P450 monooxygenases for *Arabidopsis* transformation," *Plant Biotechnology* 22, 281-286 (2005) (relating to a selection system for transformation of *Arabidopsis thaliana* via *Agrobacterium tumefaciens* that uses human cytochrome P450 monooxygenases that metabolize herbicides; herbicide tolerant seedlings were transformed and selected with the herbicides acetochlor, amiprofos-methyl, chlorpropham, chlorsulfuron, norflurazon, and pendimethalin); Siminszky et al., "Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides," *PNAS* Vol. 96, Issue 4, 1750-1755, Feb. 16, 1999; Sheldon et al, *Weed Science*: Vol. 48, No. 3, pp. 291-295, "A cytochrome P450 monooxygenase cDNA (CYP71A10) confers resistance to linuron in transgenic *Nicotiana tabacum*"; and "Phytoremediation of the herbicides atrazine and metolachlor by transgenic rice plants expressing human CYP1A1, CYP2B6, and CYP2C19," *J Agric Food Chem.* 2006 Apr. 19; 54(8):2985-91 (relating to testing a human cytochrome p450 monooxygenase in rice where the rice plants reportedly showed high tolerance to chloroacetamides (acetochlor, alachlor, metoachlor, pretilachlor, and thenylchlor), oxyacetamides (mefenacet), pyridazinones (norflurazon), 2,6-dinitroanilines (trifluralin and pendimethalin), phosphamides (amiprofos-methyl, thiocarbamates (pyributicarb), and ureas (chlortoluron)).

There is also the possibility of altering or using different 2,4-D chemistries to make the subject AAD-12 genes more efficient. Such possible changes include creating better substrates and better leaving groups (higher electronegativity).

Auxin transport inhibitors (e.g. diflufenzopyr) can also be used to increase herbicide activity with 2,4-D.

Unless specifically indicated or implied, the terms "a", "an", and "the" signify "at least one" as used herein.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

#### Example 1

##### Method for Identifying Genes That Impart Resistance to 2,4-D In Planta

As a way to identify genes which possess herbicide degrading activities in planta, it is possible to mine current public

## US 8,283,522 B2

31

databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e.,  $\alpha$ -ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with  $\geq 50\%$  homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation (relative to *tfdA* from *Ralstonia eutropha*) can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

A single gene (*sdpA*) was identified from the NCBI database (see the [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov) website; accession #AF516752) as a homologue with only 31% amino acid identity to *tfdA*. Percent identity was determined by first translating both the *sdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

## Example 2

## Optimization of Sequence for Expression in Plants and Bacteria

## 2.1—Background.

To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant

32

optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

Table 3 illustrates how high the G+C content is in maize. For the data in Table 3, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (Accelrys, San Diego, Calif.). Intron sequences were ignored in the calculations.

TABLE 3

Compilation of G + C contents  
of protein coding regions of maize genes

Protein Class <sup>a</sup>	Range % G + C	Mean % G + C <sup>b</sup>
Metabolic Enzymes (76)	44.4-75.3	59.0 (±.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (±.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (±.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (±.7.2)
All Proteins (108)	44.4-75.3	60.8 (±.5.2) <sup>c</sup>

<sup>a</sup>Number of genes in class given in parentheses.

<sup>b</sup>Standard deviations given in parentheses.

<sup>c</sup>Combined groups mean ignored in mean calculation

Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This “codon bias” is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of “minor” codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 4. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or



## US 8,283,522 B2

33

RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

TABLE 4

Preferred amino acid codons for proteins expressed in maize	
Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tyrosine	TAC/TAT
Stop	TGA/TAG

It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

34

Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Pat. No. 5,380,831.

## 2.2—AAD-12 Plant Rebuild Analysis.

Extensive analysis of the 876 base pairs (bp) of the DNA sequence of the native AAD-12 coding region (SEQ ID NO:1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO:1 (AAD-12) is presented as SEQ ID NO:2. To improve production of the recombinant protein in monocots as well as dicots, a “plant-optimized” DNA sequence AAD-12 (v1) (SEQ ID NO:3) was developed that encodes a protein (SEQ ID NO:4) which is the same as the native SEQ ID NO:2 except for the addition of an alanine residue at the second position (underlined in SEQ ID NO:4). The additional alanine codon (GCT; underlined in SEQ ID NO:3) encodes part of an NcoI restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. Thus, it serves the dual purpose of facilitating subsequent cloning operations while improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized (v1) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native and plant-optimized (v1) DNA sequences of the coding regions are only 79.7% identical. Table 5 shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences (Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

TABLE 5

Codon composition comparisons of coding regions of Native AAD-12, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.									
Amino Acid	Codon	A Native #	B Plant Opt v1 #	C Theor. Plant Opt. #	Amino Acid	Codon	D Native #	E Plant Opt v1 #	F Theor. Plant Opt. #
ALA (A)	GCA	1	10	11	LEU (L)	CTA	0	0	0
	GCC	35	16	15		CTC	1	8	8
	GCG	7	0	0		CTG	23	0	0
	GCT	0	18	17		CTT	0	8	8
ARG (R)	AGA	0	4	5	LYS (K)	TTA	0	0	0
	AGG	0	4	6		TTG	0	8	8
	CGA	0	0	0		AAA	1	1	2
	CGC	15	6	4		AAG	5	5	4
ASN (N)	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	4	3		PHE (F)	TTC	7	5
	AAC	3	2	2		TTT	1	3	3
	AAT	1	2	2		PRO (P)	CCA	0	5
ASP (D)	GAC	15	9	9		CCC	9	4	4
	GAT	2	8	8		CCG	5	0	0
CYS (C)	TGC	3	2	2		CCT	0	5	5
	TGT	0	1	1		SER (S)	AGC	5	4

US 8,283,522 B2

35

36

TABLE 5-continued

Codon composition comparisons of coding regions of Native AAD-12, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.									
Amino Acid	Codon	A Native #	B Plant Opt v1 #	C Theor. Plant Opt. #	Amino Acid	Codon	D Native #	E Plant Opt v1 #	F Theor. Plant Opt. #
END	TAA	1	0	1	AGT	0	0	0	
	TAG	0	0		TCA	0	3	3	
	TGA	0	1		TCC	2	3	3	
GLN (Q)	CAA	1	8	7	TCG	6	0	0	
	CAG	13	6	7	TCT	0	3	3	
GLU (E)	GAA	3	4	4	ACA	1	4	5	
	GAG	8	7	7	ACC	11	7	7	
GLY (G)	GGA	0	8	7	ACG	5	0	0	
	GGC	24	7	7	ACT	1	7	6	
	GGG	1	3	4	TRP (W)	TGG	8	8	8
	GGT	0	7	7	TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	9	9	TAT	1	2	2	
	CAT	8	7	7	VAL (V)	GTA	0	0	0
ILE (I)	ATA	0	2	2	GTC	6	8	7	
	ATC	10	4	5	GTG	18	8	9	
	ATT	1	5	4	GTT	0	8	8	
Totals		163	164	163	Totals		130	130	130

It is clear from examination of Table 5 that the native and plant-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The Plant-Optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the AAD-12 protein.

### 2.3 Rebuild for *E. coli* Expression

Specially engineered strains of *Escherichia coli* and associated vector systems are often used to produce relatively large amounts of proteins for biochemical and analytical studies. It is sometimes found that a native gene encoding the desired protein is not well suited for high level expression in *E. coli*, even though the source organism for the gene may be another bacterial genus. In such cases it is possible and desirable to reengineer the protein coding region of the gene to render it more suitable for expression in *E. coli*. *E. coli* Class II genes are defined as those that are highly and continuously expressed during the exponential growth phase of *E. coli* cells. (Henaut, A. and Danchin, A. (1996) in *Escherichia coli and Salmonella typhimurium cellular and molecular biology*, vol. 2, pp. 2047-2066. Neidhardt, F., Curtiss III, R., Ingraham, J., Lin, E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M. and Umberger, H. (eds.) American Society for Microbiology, Washington, D.C.). Through examination of the codon compositions of the coding regions of *E. coli* Class II genes, one can devise an average codon composition for

these *E. coli*—Class II gene coding regions. It is thought that a protein coding region having an average codon composition mimicking that of the Class II genes will be favored for expression during the exponential growth phase of *E. coli*. Using these guidelines, a new DNA sequence that encodes the AAD-12 protein (SEQ ID NO:4); including the additional alanine at the second position, as mentioned above), was designed according to the average codon composition of *E. coli* Class II gene coding regions. The initial sequence, whose design was based only on codon composition, was further engineered to include certain restriction enzyme recognition sequences suitable for cloning into *E. coli* expression vectors. Detrimental sequence features such as highly stable stemloop structures were avoided, as were intragenic sequences homologous to the 3' end of the 16S ribosomal RNA (L e. Shine Dalgarno sequences) The *E. coli*-optimized sequence (v2) is disclosed as SEQ ID NO:5 and encodes the protein disclosed in SEQ ID NO:4.

The native and *E. coli*-optimized (v2) DNA sequences are 84.0% identical, while the plant-optimized (v1) and *E. coli*-optimized (v2) DNA sequences are 76.0% identical. Table 6 presents the codon compositions of the native AAD-12 coding region (Columns A and D), an AAD-12 coding region optimized for expression in *E. coli* (v2; Columns B and E) and the codon composition of a theoretical coding region for the AAD-12 protein having an optimal codon composition of *E. coli* Class II genes (Columns C and F).

TABLE 6

Codon composition comparisons of coding regions of Native AAD-12, <i>E. coli</i> -Optimized version (v2) and a Theoretical <i>E. coli</i> Class II-Optimized version.									
Amino Acid	Codon	A Native #	B <i>E. coli</i> Opt v2 #	C Theor. Class II #	Amino Acid	Codon	D Native #	E <i>E. coli</i> Opt v2 #	F Theor. Class II #
ALA (A)	GCA	1	13	13	LEU (L)	CTA	0	0	0
	GCC	35	0	0		CTC	1	2	0
	GCG	7	18	17		CTG	23	20	24
	GCT	0	13	14		CTT	0	1	0
ARG (R)	AGA	0	0	0		TTA	0	1	0
	AGG	0	0	0		TTG	0	0	0
	CGA	0	0	0	LYS (K)	AAA	1	4	5
	CGC	15	6	6		AAG	5	2	1

US 8,283,522 B2

37

38

TABLE 6-continued

Codon composition comparisons of coding regions of Native AAD-12, <i>E. coli</i> -Optimized version (v2) and a Theoretical <i>E. coli</i> Class II-Optimized version.									
Amino Acid	Codon	A Native #	B <i>E. coli</i> Opt v2 #	C Theor. Class II #	Amino Acid	Codon	D Native #	E <i>E. coli</i> Opt v2 #	F Theor. Class II #
ASN (N)	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	12	12	PHE (F)	TTC	7	6	6
	AAC	3	4	4	PRO (P)	TTT	1	2	2
ASP (D)	AAT	1	0	0		CCA	0	3	2
	GAC	15	10	9		CCC	9	0	0
CYS (C)	GAT	2	7	8		CCG	5	11	12
	TGC	3	2	2		CCT	0	0	0
END	TGT	0	1	1	SER (S)	AGC	5	4	4
	TAA	1	1	1		AGT	0	0	0
	TAG	0	0	0		TCA	0	0	0
GLN (Q)	TGA	0	0	0		TCC	2	5	4
	CAA	1	3	3		TCG	6	0	0
	CAG	13	11	11		TCT	0	4	5
GLU (E)	GAA	3	8	8	THR (T)	ACA	1	0	0
	GAG	8	3	3		ACC	11	12	12
GLY (G)	GGA	0	0	0		ACG	5	0	0
	GGC	24	12	11		ACT	1	6	6
	GGG	1	0	0	TRP (W)	TGG	8	8	8
HIS (H)	GGT	0	13	14	TYR (Y)	TAC	4	3	3
	CAC	8	11	11		TAT	1	2	2
	CAT	8	5	5	VAL (V)	GTA	0	6	6
ILE (I)	ATA	0	0	0		GTC	6	0	0
	ATC	10	7	7		GTG	18	8	7
	ATT	1	4	4		GTT	0	10	11
Totals		163	164	164	Totals		130	130	130

35

It is clear from examination of Table 6 that the native and *E. coli*-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The *E. coli*-Optimized version (v2) closely mimics the codon composition of a theoretical *E. coli*-optimized coding region encoding the AAD-12 protein.

2.4—Design of a soybean-codon-biased DNA sequence encoding a soybean EPSPS having mutations that confer glyphosate tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is disclosed as SEQ ID NO:20 of PCT/US2005/014737 (filed May 2, 2005). A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is disclosed as SEQ ID NO:21 of PCT/US2005/014737.

A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the “kazusa.or.jp/codon” World Wide Web site. Those data were reformatted as displayed in Table 7. Columns D and H of Table 7 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 7. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

$$\text{Weighted \% of C1} = 1 / (\% \text{ C1} + \% \text{ C2} + \% \text{ C3} + \text{etc.}) \times \% \text{ C1} \times 100$$

where C1 is the codon in question, C2, C3, etc. represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of Table 7 (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of Table 7. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Ind.).

US 8,283,522 B2

39

40

TABLE 7

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.							
A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
ALA (A)	GCA	33.1	30.3	LEU (L)	CTA	DNU	9.1
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	DNU*	8.5		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARG (R)	AGA	36.0	30.9	LYS (K)	TTA	DNU	9.8
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	DNU	8.2		AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
ASN (N)	CGG	DNU	6.0	MET (M)	ATG	100.0	100
	CGT	16.9	14.5		TTC	49.2	49.2
	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0		CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1	PRO (P)	CCC	20.9	19.2
	GAT	61.9	61.9		CCG	DNU	8.3
CYS (C)	TGC	50.0	50.0	SER (S)	CCT	39.3	36.0
	TGT	50.0	50.0		AGC	16.0	15.1
END	TAA	DNU	40.7	THR (T)	AGT	18.2	17.1
	TAG	DNU	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
	CAA	55.5	55.5		TCG	DNU	6.1
GLN (Q)	CAG	44.5	44.5	TRP (W)	TCT	25.8	24.2
	GAA	50.5	50.5		ACA	32.4	29.7
GLU (E)	GAG	49.5	49.5	TYR (Y)	ACC	30.2	27.7
	GGA	31.9	31.9		ACG	DNU	8.3
GLY (G)	GGC	19.3	19.3	VAL (V)	ACT	37.4	34.3
	GGG	18.4	18.4		TGG	100.0	100
	GGT	30.4	30.4		TAC	48.2	48.2
	CAC	44.8	44.8		TAT	51.8	51.8
HIS (H)	CAT	55.2	55.2	ILE (I)	GTA	11.5	11.5
	ATA	23.4	23.4		GTC	17.8	17.8
ILE (I)	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

\*DNU = Do Not Use

To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 from PCT/US2005/014737 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21 of PCT/US2005/014737.

The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is disclosed as bases 1-1575 of SEQ ID NO:22 of PCT/US2005/014737. Synthesis of a DNA fragment comprising SEQ ID NO:22 of PCT/US2005/014737 was performed by a commercial supplier (PicoScript, Houston Tex.).

### Example 3

#### Cloning of Expression and Transformation Vectors

##### 3.1 Construction of *E. coli*, pET Expression Vector.

Using the restriction enzymes corresponding to the sites added with the additional cloning linkers (Xba 1, Xho 1) AAD-12 (v2) was cut out of the picoscript vector, and ligated into a pET280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F' *E. coli*, and plated on to Luria Broth+50 µg/ml Streptomycin & Spectinomycin (LB S/S) agar plates.

To differentiate between AAD-12 (v2): pET280 and pCR2.1: pET280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB-S/S, and grown at 37° C. for 4 hours with agitation. Each culture was then spotted onto LB+Kanamycin 50 µg/ml plates, which were incubated at 37° C. overnight. Colonies that grew on the LB-K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before, and checked for correctness with digestion by XbaI/XhoI. The final expression construct was given the designation pDAB3222.

##### 3.2—Construction of *Pseudomonas* Expression Vector

The AAD-12 (v2) open reading frame was initially cloned into the modified pET expression vector (Novagen), "pET280 S/S", as an XbaI-XhoI fragment. The resulting plasmid pDAB725 was confirmed with restriction enzyme digestion and sequencing reactions. The AAD-12 (v2) open reading frame from pDAB725 was transferred into the *Pseudomonas*



## US 8,283,522 B2

41

expression vector, pMYC1803, as an XbaI-XhoI fragment. Positive colonies were confirmed via restriction enzyme digestion. The completed construct pDAB739 was transformed into the MB217 and MB324 *Pseudomonas* expression strains.

## 3.3—Completion of Binary Vectors.

The plant optimized gene AAD-12 (v1) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:3) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:6) and M13 Reverse (SEQ ID NO:7) primers using the Beckman Coulter “Dye Terminator Cycle Sequencing with Quick Start Kit” reagents as before. Sequence data

42

ing binary vector, pDAB724, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: AAD-12 (v1): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR] was restriction digested (with Bam HI, Nco I, Not I, SacI, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB724) was used for transformation into *Agrobacterium* (see section 7.2).

## 3.4—Cloning of Additional Transformation Constructs.

All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular cloning methods (Maniatis et al., 1982). Table 8 lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

TABLE 8

Binary constructs used in transformation of various plant species.													
pDAB #	pDAS #	Species* Trans-formed into	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
724	—	A, Ct, S	AAD12 v1	AtUbi10	NtOsm	—	—	—	Erythromycin	—	pat	CsVMV	Agro binary
3274	—	A	AAD12 v1	AtUbi10	NtOsm	RB7 Mar v2	—	—	Spectinomycin	—	—	—	Agro binary
3278	1580	T	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	—	—	Spectinomycin	—	pat	AtUbi10	Agro binary
3285	—	A	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	—	—	Spectinomycin	—	pat	AtUbi10	Agro binary
3759	—	A, Ca, S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectinomycin	—	pat	AtUbi10	Agro binary
4101	1863	Cn, R	AAD12 v1	ZmUbi1	—	RB7 Mar v2	—	—	Ampicillin	—	AHAS v3	OsAct1	Whiskers/ binary Gun
4464	—	S	AAD12 v1	CsVMV	—	RB7 Mar v2	—	—	Spectinomycin	—	pat	CsVMV	Agro binary
4468	—	S	AAD12 v1	AtUbi10	—	RB7 Mar v2	—	—	Spectinomycin	—	pat	CsVMV	Agro binary
4472	—	S	AAD12 v1	AtUbi3	—	RB7 Mar v2	—	—	Spectinomycin	—	pat	CsVMV	Agro binary
4476	—	S	AAD12 v1	ZmUbi1	—	RB7 Mar v2	—	—	Spectinomycin	—	pat	CsVMV	Agro binary
4480	—	S	AAD12 v1	AtAct2	—	RB7 Mar v2	—	—	Spectinomycin	—	pat	CsVMV	Agro binary

\*A = Arabidopsis

T = Tobacco

S = Soybean

Ct = Cotton

R = Rice

Cn = Corn

Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter

AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 PromoterAtUbi3 = *Arabidopsis thaliana* Ubiquitin 3 PromoterAtAct2 = *Arabidopsis thaliana* Actin 2 PromoterRB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated RegionZmUbi1 = *Zea mays* Ubiquitin 1 Promoter

HptII = hygromycin phosphotransferase

was analyzed and results indicated that no anomalies were present in the plant optimized AAD-12 (v1) DNA sequence. The AAD-12 (v1) gene was cloned into pDAB726 as an Nco I-Sac I fragment. The resulting construct was designated pDAB723, containing: [AtUbi10 promoter: Nt OSM 5'UTR: AAD-12 (v1): Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with a PvuII and a Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The result-

## Example 4

Recombinant AAD-12 (v2) Expression and Purification in *Pseudomonas fluorescens*4.1—*Pseudomonas fluorescens* Fermentation

For shake flask experiment, 200 µl of *Pseudomonas fluorescens* strain MB324 glycerol stock carried AAD-12 (v2) construct pDAB739 (sec 3.2) was used to inoculate 50 ml

US 8,283,522 B2

43

fresh LB media supplemented with 30 µg/ml tetracycline/HCl. The culture (in a 250 ml baffled Erlenmeyer flask) was incubated on a shaker (New Brunswick Scientific Model Innova 44) at 300 rpm and 30° C. for 16 hrs. 20 ml of seed culture was transferred into 1 L *Pseudomonas fluorescens* culture media (Yeast extract, 5 g/L; K<sub>2</sub>HPO<sub>4</sub>, 5 g/L; (NH<sub>4</sub>)<sub>2</sub>PO<sub>4</sub>, 7.5 g/L; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; MgSO<sub>4</sub>—7H<sub>2</sub>O, 1 g/L; KCl, 0.5 g/L; CaCl<sub>2</sub>—2H<sub>2</sub>O, 0.5 g/L; NaCitrate—2H<sub>2</sub>O, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 ml/L; Trace element solution: FeCl<sub>3</sub>—6H<sub>2</sub>O, 5.4 g/L; MnCl<sub>2</sub>—4H<sub>2</sub>O, 1 g/L; ZnSO<sub>4</sub>—7H<sub>2</sub>O, 1.45 g/L; CuSO<sub>4</sub>—5H<sub>2</sub>O, 0.25 g/L; H<sub>3</sub>BO<sub>3</sub>, 0.1 g/L; (NH<sub>4</sub>)<sub>6</sub>MO<sub>7</sub>O<sub>24</sub>, 0.1 g/L; concentrated HCl, 13 ml/L) supplemented with 20 tetracycline/HCl and 250 µl of Pluronic L61 (anti-foam) in a 2.8 L baffled Erlenmeyer flask. The cultures were incubated at 30° C. and 300 rpm for 24 hrs. Isopropyl β-D-1-thiogalacto-pyranoside (IPTG) was added to 1 mM final in the cultures and continued to incubate for approximately 48 hrs at 25° C. Cells were harvested by centrifugation at 7 krpm at 4° C. for 15 min, and cell paste was stored at -80° C. or immediately processed for purification.

For tank experiments, 1 ml each of the glycerol stock was inoculated a 1 L baffled flask containing 200 ml of LB media supplemented with 30 µg/ml tetracycline/HCl at 300 rpm and 32° C. for 16-24 hrs. The combined culture from three flasks (600 ml) was then aseptically transferred to a 20 L fermentor (B. Braun Bioreactor Systems) containing 10 L of Dow proprietary defined medium (through Teknova, Hollister, Calif.) designed to support high cell density growth. Growth temperature was maintained at 32° C. and the pH was controlled at the desired set-point through the addition of aqueous ammonia. Dissolved oxygen was maintained at a positive level in the liquid culture by regulating the sparged air flow and the agitation rates. The fed-batch fermentation process was carried out for approximately 24 hrs till cell density reached 170-200 OD<sub>575</sub>. IPTG was added to 1 mM to induce the recombinant protein expression and the temperature was reduced and maintained to 25° C. using circulation of cold-water supply. The induction phase of the fermentation was allowed to continue for another 24 hrs. Samples (30 ml) were collected for various analyses to determine cell density and protein expression level at 6, 12, and 18 hrs post-induction time points. At the end of a fermentation run, cells were harvested by centrifugation at 10 krpm for 30 min. The cell pellets were frozen at -80° C. for further processing.

#### 4.2—Purification of AAD-12 (v2) for Biochemical Characterization and Antibody Production

Approximately 100-200 g of frozen (or fresh) *Pseudomonas* cells were thawed and resuspended in 1-2 L of extraction buffer containing 20 mM Tris-HCl, pH 8.5, and 25 ml of Protease inhibitor cocktail (Sigma cat#P8465). The cells were disrupted using Microfluidizer (model M110L or 110Y) (Microfluidics, Newton, Mass.) on ice with one pass at 11,000-12,000 psi. The lysate was centrifuged at 24,000 rpm for 20 min. The supernatant was transferred and dialyzed against 10 volumes of 20 mM Tris-HCl, pH 8.5 overnight at 4° C., or diafiltered with this buffer and filtered through a 0.45 µm membrane before applying to the column separations. All subsequent protein separations were performed using Pharmacia AKTA Explorer 100 and operated at 4° C. Prior to loading, a Q Sepharose Fast Flow column (Pharmacia XK 50/00, 500 ml bed size) was equilibrated with 20 mM Tris-HCl, pH 8.5 buffer. The sample was applied to the column at 15 ml/min and then washed with this buffer until the eluate OD<sub>280</sub> returned to baseline. Proteins were eluted with

44

2 L of linear gradient from 0 to 0.3 M NaCl at a flow rate of 15 ml/min, while 45 ml fractions were collected. Fractions containing AAD-12 activity as determined by the colorimetric enzyme assay and also corresponding to the predicted molecular weight of AAD-12 protein (about 32 kDa band on SDS-PAGE) were pooled. Solid ammonium sulfate to final 0.5 M was added to the sample, and then applied to a Phenyl HP column (Pharmacia XK 50/20, 250 ml bed size) equilibrated in 0.5 M ammonium sulfate in 20 mM Tris-HCl, pH 8.0. This column was washed with the binding buffer at 10 ml/min until the OD<sub>280</sub> of the eluate returned to baseline, proteins were eluted within 2 column volumes at 10 ml/min by a linear gradient from 0.5 M to 0 M Ammonium sulfate in 20 mM Tris-HCl, pH 8.0, and 12.5 ml fractions were collected. The main peak fractions containing AAD-12 were pooled, and if necessary, concentrated using a MWCO 10 kDa cut-off membrane centrifugal filter device (Millipore). In some cases the sample was further applied to a Superdex 75 gel filtration column (Pharmacia XK 16/60, 110 ml bed size) with PBS buffer at a flow rate of 1 ml/min. Peak fractions containing pure AAD-12 were pooled and stored at -80° C. In most cases, AAD-12 protein purity is approaching or above 99% after sequential ion-exchange column and hydrophobic interaction column two-step separation. A typical yield for purified AAD-12 is 12-18 mg/g of wet cells. Bulk protein sample was formulated in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM DTT, and 1% Trehalose by diafiltration, and lyophilized on the Virtis Freezemobile Model 25EL (Virtis, Gardiner, N.Y.) for long-term storage.

Protein concentration was initially measured by Bradford assay using Bio-Rad Protein assay kit (cat#500-0006) with bovine serum albumin as standard. When needed, more accurate protein concentration was determined by using total amino acid hydrolysis. The sample was analyzed in Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, Calif.) with amino acid calibration standards (cat#PN5061-3330) purchased from Agilent.

AAD-12 activity was determined through out the processes to ensure no loss of the enzyme activity by each treatment and manipulation, as described in the Example 5 below. Protein purity was monitored by using SDS-PAGE and analytical size exclusion chromatography. Purified protein sample was further verified and confirmed by N-terminal amino acid sequencing, and shown consisting of expected AQTTLQITPT residues at its N-terminus. Short and long-term protein stability was tested by enzymatic activity and by native-PAGE and SDS-PAGE gel analysis under both non-reducing and reducing conditions. And it was noticed that AAD-12 is prone to oligomerization via disulfide bond formation, therefore typically 2 mM DTT was used for protein storage. Phosphate-buffer saline (PBS) and Tris-buffer saline (TBS) were tested for protein lyophilization, with and without the presence of 1% trehalose. Additionally, the endotoxin and DNA contaminant context from purified sample were measured respectively, and the integrity of the AAD-12 protein was also assessed by isoelectric focusing (IEF) analysis.

Ten milligrams of purified AAD-12 (v2) was delivered to Zymed Laboratories, Inc. (South San Francisco, Calif.) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 ml of complete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification, and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

## US 8,283,522 B2

45

## Example 5

## In Vitro Assays of AAD-12 Activity

## 5.1—Assay Via Colorimetric Phenol Detection.

Enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100  $\mu\text{M}$  in 0.15 ml 20 mM MOPS pH 6.75 containing 200  $\mu\text{M}$   $\text{NH}_4(\text{FeSO}_4)_2$ , 200  $\mu\text{M}$  sodium ascorbate. Pyridinols derived from fluoroxypr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol was linear and proportional to the concentration of phenol in the assay up to  $\sim 500 \mu\text{M}$ . A calibration curve performed under standard assay conditions (160  $\mu\text{l}$  final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 17.2  $\mu\text{M}$  phenol.

Enzyme assays were performed in a total volume of 0.16 ml 20 mM MOPS pH 6.75 containing 200  $\mu\text{M}$   $\text{NH}_4\text{FeSO}_4$ , 200  $\mu\text{M}$  sodium ascorbate, 1 mM  $\alpha$ -ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or  $\alpha$ -ketoglutarate at time zero. After 5 minutes of incubation at 25°C., the reaction was terminated by addition of 30  $\mu\text{l}$  of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer (3.09 g boric acid+3.73 g KCl+44 ml 1 N KOH) and 0.2% 4-aminoantipyrine. Then 10  $\mu\text{l}$  0.8% potassium ferricyanide was added and after 5 or 10 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols.

## 5.2—Assay Via Detection of Chloropyridinol

AAD-12 action on potential substrates such as the herbicide triclopyr containing a substituted pyridine (rather than benzene rings) will release a pyridinol on cleavage of the aryloxyalkanoate bond. Pyridinols were not detected using the aminoantipyrine/ferricyanide phenol detection described in the preceding section. However, it was found that product chloropyridinols absorb strongly in the near UV with  $\lambda_{\text{max}}$  of 325 nm at pH 7 (extinction coefficient  $\sim 8,400 \text{ M}^{-1}\text{cm}^{-1}$ ). This was used to create a continuous microplate-based spectrophotometric assay. Assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200  $\mu\text{M}$   $\text{NH}_4\text{FeSO}_4$ , 200  $\mu\text{M}$  sodium ascorbate, 1 mM  $\alpha$ -ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or  $\alpha$ -ketoglutarate at time zero and the increase in absorbance followed for 10 minutes at 325 nm in a microplate reader. The first 2 minutes of the reaction was used to determine initial rates. A calibration curve performed under standard assay

46

conditions (200  $\mu\text{l}$  final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9  $\mu\text{M}$  chloropyridinol.

## 5.3—Colorimetric assay using 2-(2-chloro,4-nitrophenoxy)propionate

A convenient assay of AAD-12 was devised using 2-(2-chloro,4-nitrophenoxy)propionate (CNPP) as substrate. Cleavage of CNPP by AAD-12 releases 2-chloro,4-nitrophenol. This phenol has a bright yellow absorbance at 410 nm at pH 7 enabling the reaction to be followed continuously or by endpoint analysis. The presence of AAD-12 activity can be monitored visually without the need for addition of further reagents. Microplate-based spectrophotometric assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200  $\mu\text{M}$   $\text{NH}_4\text{FeSO}_4$ , 200  $\mu\text{M}$  sodium ascorbate, 1 mM  $\alpha$ -ketoglutarate, the appropriate amount of CNPP (added from a 10 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of CNPP, enzyme, or  $\alpha$ -ketoglutarate at time zero and the increase in absorbance followed for 10 min at 410 nm in a microplate reader. The first 2 min of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200  $\mu\text{l}$  final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1  $\mu\text{M}$  2-chloro, 4-nitrophenol. Using this assay, the kinetic constants for CNPP as a substrate were determined to be  $K_m = 31 \pm 5.5 \mu\text{M}$  and  $k_{\text{cat}} = 16.2 \pm 0.79 \text{ min}^{-1}$ .

## Example 6

## In Vitro Activity of AAD-12 on Various Substrates

## 6.1—AAD-12 (v2) activity on (R,S)— dichlorprop, (R)—dichlorprop, (S)—dichlorprop and 2,4-D

Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4  $\mu\text{g}$  purified AAD-12 (v2). (R,S)—dichlorprop (R,S-DP) was tested at 1 mM and (R)—dichlorprop, (S)—dichlorprop and 2,4-D were tested at 0.5 mM. The results are shown in FIG. 3, which illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop. 4.4  $\mu\text{g}$  AAD-12 (v2) was incubated with 0.5 mM substrate (1 mM for (R,S)—dichlorprop) and the reaction initiated by addition of  $\alpha$ -ketoglutarate. After 5 minutes, the reaction was quenched, and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.

AAD-12 (v2) has excellent activity on (R,S)—dichlorprop and (S)—dichlorprop and has minimal activity on (R)—dichlorprop. This indicates that AAD-12 (v2) has a clear (S)—enantiomeric preference. The activity of AAD-12 (v2) on 2,4-D was equivalent to that on (S)—dichlorprop indicating that the enzyme can process oxypropionate and oxyacetates effectively.

## 6.2—AAD-12 (v2) Activity on Pyridyloxyalkanoates

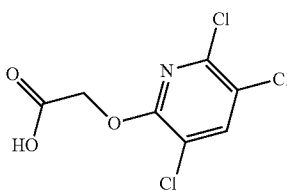
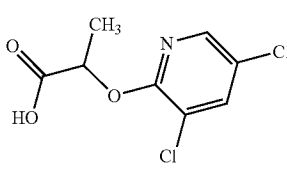
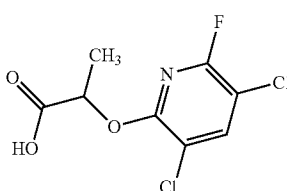
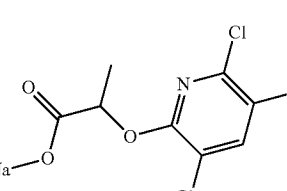
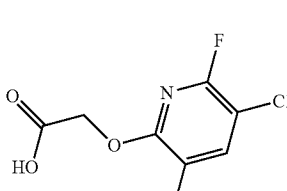
Using the pyridinol assay described in Example 5.2, five pyridyloxyalkanoates were assayed at 1 mM in a reaction mix containing 6.8  $\mu\text{g}$  purified AAD-12 (v2). The rates of each reaction were monitored and are presented in Table 9. All five pyridyloxyalkanoates were cleaved to release pyridinols by AAD-12 (v2). The rates for the oxypropionate substrates 116844 and 91767 were somewhat faster than those for the corresponding acetates (triclopyr and 93833 respectively) indicating a preference of AAD-12 (v2) for oxypropionate over oxyacetate side chains. These data show that AAD-12 (v2) is able to effectively degrade pyridyloxyalkanoate herbicides such as triclopyr.

## US 8,283,522 B2

47

TABLE 9

Rates of pyridyloxyalkanoate cleavage by AAD-12 (v2). 6.8  $\mu$ g AAD-12 (v2) was incubated with 1 mM substrate, the reaction initiated by addition of  $\alpha$ -ketoglutarate and the subsequent increase in absorbance monitored at 325 nm. The background rate of 1.4 mAU/min without  $\alpha$ -ketoglutarate was subtracted from the rates with substrate.

STRUCTURE	ID	Rate (mAU/ min)	Rate relative to triclopyr
	triclopyr	97	1
	66357	225	2.3
	91767	190	0.8
	116844	257	1.4
	93833	118	0.5

### 6.3—Kinetic Constants of AAD-12 (v2) for 2,4-D, (R,S)-DCP and Triclopyr

The  $K_m$  and  $k_{cat}$  values of purified AAD-12 (v2) for the herbicides 2,4-D, (R,S)-dichlorprop and triclopyr were determined using the appropriate assay method. Substrate inhibition occurred at high concentrations ( $>1$  mM) of 2,4-D and (R,S)-DCP so concentrations below this were used to fit the data to the Michaelis-Menten equation using Grafit 4.0 (Erithacus Software, UK). No substrate inhibition was noted for triclopyr up to 2 mM. The kinetic constants are summarized in Table 10. From these data, the rate of AAD-12 (v2) cleavage of triclopyr is  $\sim 5\%$  that of 2,4-D, under maximal velocity conditions.

48

TABLE 10

Kinetic constants of AAD-12 (v2) for three herbicide substrates				
Substrate	$K_m$ , $\mu$ M ( $\pm$ SE)	$k_{cat}$ , $\text{min}^{-1}$ ( $\pm$ SE)	Assay method	Substrate inhibition at 2 mM
2,4-D	102 ( $\pm 18.4$ )	54.1 ( $\pm 3.1$ )	Phenol detection	55%
(R,S)- dichlorprop	122 ( $\pm 2.7$ )*	63.4 ( $\pm 0.5$ )	Phenol detection	55%
Triclopyr	241 ( $\pm 30$ )	2.6 ( $\pm 0.1$ )	$\Delta A_{325 \text{ nm}}$	0%

\*Because of the (S)-enantiomeric preference of AAD-12, the  $K_m$  value was calculated assuming 50% of the racemic mixture was available as a substrate

### Example 7

#### Transformation into *Arabidopsis* and Selection

##### 7.1—*Arabidopsis thaliana* Growth Conditions.

Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, Mo.) solution. The suspended seed was stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, Wash.) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150  $\mu\text{mol/m}^2 \text{ sec}$  under constant temperature (22° C.) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

##### 7.2—*Agrobacterium* Transformation.

An LB+agar plate with erythromycin (Sigma Chemical Co., St. Louis, Mo.) (200 mg/L) or spectinomycin (100 mg/L) containing a streaked DH5 $\alpha$  colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB+erythromycin). The cultures were incubated overnight at 37° C. with constant agitation. Qiagen (Valencia, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50  $\mu$ l of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4 kV, Pulse length: 5 msec.

After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C. in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, Mo.) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

Colonies were selected and streaked onto fresh YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28° C.



for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP+erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed *Agrobacterium* cells, suspended in 10  $\mu$ l of water, at 100° C. for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wis.) per manufacturer's instructions at 0.5 $\times$  concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C. for 3 minutes, 2) 94° C. for 45 seconds, 3) 55° C. for 30 seconds, 4) 72° C. for 1 minute, for 29 cycles then 1 cycle of 72° C. for 10 minutes. The reaction was maintained at 4° C. after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

### 7.3—*Arabidopsis* Transformation.

*Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C. with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C. with constant agitation. The cells were then pelleted at approx. 8700 $\times$ g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing:  $\frac{1}{2}\times$  Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044  $\mu$ M benzylamino purine (10  $\mu$ l/liter of 1 mg/ml stock in DMSO) and 300  $\mu$ l/liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C., with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

### 7.4—Selection of Transformed Plants.

Freshly harvested  $T_1$  seed [AAD-12 (v1) gene] was allowed to dry for 7 days at room temperature.  $T_1$  seed was sown in 26.5 $\times$ 51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified  $T_1$  seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed PAT gene).

Seven days after planting (DAP) and again 11 DAP,  $T_1$  plants (cotyledon and 2-4-1f stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22 $\pm$ 5° C., 50 $\pm$ 30% RH, 14 h light:10 dark, minimum 500  $\mu$ E/m<sup>2</sup> s<sup>-1</sup> natural+supplemental light) at least 1 day prior to testing for the ability of AAD-12 (v1) (plant optimized gene) to provide phenoxy auxin herbicide resistance.

$T_1$  plants were then randomly assigned to various rates of 2,4-D. For *Arabidopsis*, 50 g ae/ha 2,4-D is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (50, 200, 800, or 3200 g ae/ha). Tables 10 and 11 show comparisons drawn to an aryloxyalkanoate herbicide resistance gene (AAD-1 (v3)) previously described in PCT/US2005/014737.

All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.). Dichlorprop used was commercial grade formulated as potassium salt of R-dichlorprop (600 g ai/L, AH Marks). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of herbicides in 200 mM HEPES buffer, pH 7.5.

Some  $T_1$  individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether the pyridyloxyacetate auxin herbicides, triclopyr and fluoroxypyr, could be effectively degraded in planta. Herbicides were applied to  $T_1$  plants with use of a track sparyer in a 187 L/ha spray volume.  $T_1$  plants that exhibited tolerance to 2,4-D DMA were further accessed in the  $T_2$  generation.

### 7.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-12 (v1) (plant optimized gene).  $T_1$  transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000  $T_1$  seed were screened and 316 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.10% which lies in the normal range of selection frequency of constructs where PAT+Liberty are used for selection.  $T_1$  plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 11 compares the response of AAD-12 (v1) and control genes to impart 2,4-D resistance to *Arabidopsis*  $T_1$  transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each  $T_1$  is an independent transformation

## US 8,283,522 B2

## 51

event, one can expect significant variation of individual  $T_1$  responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The AAD-12 (v1) gene imparted herbicide resistance to individual  $T_1$  *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 11 simply to demonstrate the significant difference between the plants transformed with AAD-12 (v1) versus the wildtype or PAT/Cry1F-transformed controls. Injury levels tend to be greater and the frequency of uninjured plants was lower at elevated rates up to 3,200 g ae/ha (or 6× field rate). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, many individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

TABLE 11

AAD-12 (v1) transformed $T_1$ <i>Arabidopsis</i> response to a range of 2,4-D rates applied postemergence, compared to an AAD-1 v3 ( $T_4$ ) homozygous resistant population, or a Pat-Cry1F transformed, auxin-sensitive control.					
	% Injury			% Injury	Std
	<20%	20-40%	>40%	Ave	Dev
AAD-12 (v1) gene $T_1$ transformants Averages					
Untreated control-buffer	6	0	0	0	0
50 g ae/ha 2,4-D	6	0	2	16	24
200 g ae/ha 2,4-D	6	1	1	11	18
800 g ae/ha 2,4-D	5	2	1	15	20
3200 g ae/ha 2,4-D	8	0	0	6	6
PAT/Cry1F (transformed control) Averages					
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	4	1	5	31	16
200 g ae/ha 2,4-D	0	0	10	70	2
800 g ae/ha 2,4-D	0	0	10	81	8
3200 g ae/ha 2,4-D	0	0	10	91	2
Homozygous AAD-1 (v3) gene $T_4$ plants Averages					
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	10	0	0	0	0
200 g ae/ha 2,4-D	10	0	0	0	0
800 g ae/ha 2,4-D	10	0	0	0	0
3200 g ae/ha 2,4-D	9	1	0	2	6

Table 12 shows a similarly conducted dose response of  $T_1$  *Arabidopsis* to the phenoxypyropionic acid, dichlorprop. The data shows that the herbicidally active (R—) isomer of dichlorprop does not serve as a suitable substrate for AAD-12 (v1). The fact that AAD-1 will metabolize R-dichlorprop well enough to impart commercially acceptable tolerance is one distinguishing characteristic that separates the two genes. (Table 12). AAD-1 and AAD-12 are considered R- and S-specific  $\alpha$ -ketoglutarate dioxygenases, respectively.

## 52

TABLE 12

$T_1$ <i>Arabidopsis</i> response to a range of R-dichlorprop rates applied postemergence.					
	% Injury			% Injury	Std
	<20%	20-40%	>40%	Ave	Dev
AAD-12 v1 gene Averages					
Untreated control	6	0	0	0	0
50 g ae/ha R-dichlorprop	0	0	8	63	7
200 g ae/ha R-dichlorprop	0	0	8	85	10
800 g ae/ha R-dichlorprop	0	0	8	96	4
3200 g ae/ha R-dichlorprop	0	0	8	98	2
PAT/Cry1F Averages					
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	0	10	0	27	2
200 g ae/ha R-dichlorprop	0	0	10	69	3
800 g ae/ha R-dichlorprop	0	0	10	83	6
3200 g ae/ha R-dichlorprop	0	0	10	90	2
Homozygous AAD-1 (v3) gene $T_4$ plants					
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	10	0	0	0	0
200 g ae/ha R-dichlorprop	10	0	0	0	0
800 g ae/ha R-dichlorprop	10	0	0	0	0
3200 g ae/ha R-dichlorprop	10	0	0	0	0

## 7.6—AAD-12 (v1) as a Selectable Marker.

The ability to use AAD-12 (v1) as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed as described above. Approximately 50  $T_4$  generation *Arabidopsis* seed (homozygous for AAD-12 (v1)) were spiked into approximately 5,000 wildtype (sensitive) seed. Several treatments were compared, each tray of plants receiving either one or two application timings of 2,4-D in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 followed by 11 DAP. Since all individuals also contained the PAT gene in the same transformation vector, AAD-12 selected with 2,4-D could be directly compared to PAT selected with glufosinate.

Treatments were applied with a DeVilbiss spray tip as previously described. Plants were identified as Resistant or Sensitive 17 DAP. The optimum treatment was 75 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), was equally effective in selection frequency, and resulted in less herbicidal injury to the transformed individuals than the Liberty selection scheme. These results indicate AAD-12 (v1) can be effectively used as an alternative selectable marker for a population of transformed *Arabidopsis*.

## 7.7—Heritability.

A variety of  $T_1$  events were self-pollinated to produce  $T_2$  seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random  $T_2$  siblings. Each individual  $T_2$  plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). Seventy-five percent of the  $T_1$  families ( $T_2$  plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ( $P>0.05$ ).

Seed were collected from 12 to 20  $T_2$  individuals ( $T_3$  seed). Twenty-five  $T_3$  siblings from each of eight randomly-selected  $T_2$  families were progeny tested as previously described. Approximately one-third of the  $T_2$  families anticipated to be homozygous (non-segregating populations) have been identified in each line. These data show AAD-12 (v1) is stably integrated and inherited in a Mendelian fashion to at least three generations.

## US 8,283,522 B2

53

7.8—Additional Foliar Applications Herbicide Resistance in AAD-12 *Arabidopsis*.

The ability of AAD-12 (v1) to provide resistance to other aryloxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates. T<sub>2</sub> generation *Arabidopsis* seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed with a range of pyridyloxyacetate herbicides: 280-2240 g ae/ha triclopyr (Garlon 3A, Dow AgroSciences) and 280-2240 g ae/ha fluroxypyr (Starane, Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-12 activity, 2,4-dichlorophenol (DCP, Sigma) (at a molar equivalent to 280-2240 g ae/ha of 2,4-D, technical grade DCP was used). All applications were formulated in water. Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment.

There is no effect of the 2,4-D metabolite, 2,4-dichlorophenol (DCP), on transgenic non-AAD-12 control *Arabidopsis* (Pat/Cry1F). AAD-12-transformed plants were also clearly protected from the triclopyr and fluroxypyr herbicide injury that was seen in the transformed non-resistant controls (see Table 13). These results confirm that AAD-12 (v1) in *Arabidopsis* provides resistance to the pyridyloxyacetic auxins tested. This is the first report of an enzyme with significant activity on pyridyloxyacetic acid herbicides. No other 2,4-D degrading enzyme has been reported with similar activity.

TABLE 13

Comparison of T <sub>2</sub> AAD-12 (v1) and transformed control <i>Arabidopsis</i> plant response to various foliar-applied auxinic herbicides. Pyridyloxyacetic auxins		
Ave % Injury 14DAT		
Herbicide Treatment	Segregating T <sub>2</sub> AAD-12 (v1) plants (pDAB724.01.120)	Pat/Cry1F-Control
280 g ae/ha Triclopyr	0	52
560 g ae/ha Triclopyr	3	58
1120 g ae/ha Triclopyr	0	75*
2240 g ae/ha Triclopyr	3	75*
280 g ae/ha Fluroxypyr	0	75*
560 g ae/ha Fluroxypyr	2	75*
1120 g ae/ha Fluroxypyr	3	75*
2240 g ae/ha Fluroxypyr	5	75*
Inactive DCP metabolite		
280 g ae/ha 2,4-DCP	0	0
560 g ae/ha 2,4-DCP	0	0
1120 g ae/ha 2,4-DCP	0	0
2240 g ae/ha 2,4-DCP	0	0

\*Plants in this experiment were stunted and severely epinastic, but remained green and did not receive injury ratings >75%.

7.9—Molecular Analysis of AAD-12 (v1) *Arabidopsis*.

Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple AAD-12 (v1) homozygous lines to determine stable integration of the plant transformation unit containing PAT and AAD-12 (v1). Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

Results showed that all 2,4-D resistant plants assayed, contained PAT (and thus by inference, AAD-12 (v1)). Copy number analysis showed total inserts ranged from 1 to 5 copies. This correlates, too, with the AAD-12 (v1) protein expression data indicating that the presence of the enzyme

54

yields significantly high levels of resistance to all commercially available phenoxyacetic and pyridyloxyacetic acids.

7.10—*Arabidopsis* Transformed with Molecular Stack of AAD-12 (v1) and a Glyphosate Resistance Gene.

T<sub>1</sub> *Arabidopsis* seed was produced, as previously described, containing the pDAB3759 plasmid (AAD-12 (v1)+EPSPS) which encodes a putative glyphosate resistance trait. T<sub>1</sub> transformants were selected using AAD-12 (v1) as the selectable marker as described in example 7.6. T<sub>1</sub> plants (individually transformed events) were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, AAD-12 (v1)+PAT T<sub>4</sub> homozygous lines (pDAB724-transformed), and PAT+Cry1F homozygous line (transformed control). The pDAB3759 and pDAB724 transformed plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in water. All treatments were replicated 5 to 20 times. Plants were evaluated 7 and 14 days after treatment.

Initial resistance assessment indicated plants tolerant to 2,4-D were subsequently tolerant to glyphosate when compared to the response of the three control lines. These results indicate that resistance can be imparted to plants to two herbicides with differing modes of action, including 2,4-D and glyphosate tolerance, allowing application of both herbicides postemergence. Additionally, AAD-12+2,4-D was used effectively as a selectable marker for a true resistance selection.

TABLE 14

T <sub>1</sub> <i>Arabidopsis</i> response to a range of glyphosate rates applied postemergence (14 DAT).					
	% Injury			% Injury	Std
	<20%	20-40%	>40%	Ave	Dev
AAD-12 v1 gene + EPSPS + HptII (pDAB3759) (Averages)					
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	13	2	1	11	16
105 g ae/ha glyphosate	10	1	5	34	38
420 g ae/ha glyphosate	5	6	5	44	37
1680 g ae/ha glyphosate	0	0	16	85	9
PAT/Cry1F Averages					
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	67	7
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0
Wildtype (Col-0) Averages					
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	75	13
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0
pDAB724 T <sub>4</sub> (PAT + AAD-12) Averages					
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	66	8
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate 0	0	0	5	100	0



7.11—AAD-12 *Arabidopsis* Genetically Stacked with AAD-1 to Give Wider Spectrum of Herbicide Tolerance.

AAD-12 (v1) (pDAB724) and AAD-1 (v3) (pDAB721) plants were reciprocally crossed and F<sub>1</sub> seed was collected. Eight F<sub>1</sub> seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F<sub>1</sub> plants and subjected to Western analysis to confirm the presence of both genes. It was concluded that all 8 plants tested expressed both AAD-1 and AAD-12 proteins. The seed was bulked and allowed to dry for a week before planting.

One hundred F<sub>2</sub> seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F<sub>2</sub> plants survived glufosinate selection fitting an expected segregation ratio for two independently assorting loci for glufosinate resistance (15 R:1 S). Glufosinate resistant plants were then treated with 560 g ae/ha R-dichlorprop+560 g ae/ha triclopyr, applied to the plants under the same spray regimen as used for the other testing. Plants were graded at 3 and 14 DAT. Sixty-three of the 96 plants that survived glufosinate selection also survived the herbicide application. These data are consistent with an expected segregation pattern (9R: 6S) of two independently assorting dominant traits where each gene gives resistance to only one of the auxinic herbicides (either R-dichlorprop or triclopyr). The results indicate that AAD-12 (pDAB724) can be successfully stacked with AAD-1 (pDAB721), thus increasing the spectrum herbicides that may be applied to the crop of interest [(2,4-D+R-dichlorprop) and (2,4-D+fluoroxypyr+triclopyr), respectively]. This could be useful to bring 2,4-D tolerance to a very sensitive species through conventional stacking of two separate 2,4-D resistance genes. Additionally, if either gene were used as a selectable marker for a third and fourth gene of interest through independent transformation activities, then each gene pair could be brought together through conventional breeding activities and subsequently selected in the F<sub>1</sub> generation through paired sprays with herbicides that are exclusive between the AAD-1 and AAD-12 enzymes (as shown with R-dichlorprop and triclopyr for AAD-1 and AAD-12, respectively, above).

Other AAD stacks are also within the scope of the subject invention. The TfdA protein discussed elsewhere herein (Streber et al.), for example, can be used together with the subject AAD-12 genes to impart novel spectrums of herbicide resistance in transgenic plants of the subject invention.

#### Example 8

##### WHISKERS-Mediated Transformation of Corn Using Imazethapyr Selection

###### 8.1—Cloning of AAD-12 (v1).

The AAD-12 (v1) gene was cut out of the intermediate vector pDAB3283 as an NcoI/SacI fragment. This was ligated directionally into the similarly cut pDAB3403 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5 $\alpha$  cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. This first intermediate construct (pDAB4100) contains the ZmUbi1: AAD-12 (v1) cassette. This construct was digested with NotI and PvuI to liberate the gene cassette and digest the unwanted backbone. This was ligated to NotI cut pDAB2212, which contains the AHAS selectable marker driven by the Rice Actin promoter OsAct1. The final construct was designated pDAB4101 or pDAS1863, and contains ZmUbi1/AAD-12 (v1)/ZmPer5::OsAct1/AHAS/LZmLip.

###### 8.2—Callus/Suspension Initiation.

To obtain immature embryos for callus culture initiation, F<sub>1</sub> crosses between greenhouse-grown Hi-11 parents A and B (Armstrong et al. 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu et al., 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L AgNO<sub>3</sub>, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter et al., 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L  $\alpha$ -naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

###### 8.3—Cryopreservation and Thawing Of Suspensions.

Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C. for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C. in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C./minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Form a Scientific) filled with liquid nitrogen vapors.

For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C. until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off

the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 ml H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

#### 8.4—Stable Transformation

Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L myo-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker shaken at 125 RPM in the dark for 30-35 minutes at 28° C., and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume 8.1 ml of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, sterile silicon carbide whiskers (Advanced Composite Materials, Inc.).

After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~44 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was then added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker at 125 RPM for 2 hours at 28° C. before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

Approximately 2 ml of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60x20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C. in a dark box.

After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (3P) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 3 µM imazethapyr from Pursuit® DG, 2.5 g/L Gelrite, pH 5.8). Plates were placed in boxes and cultured for an additional week.

Two weeks post-transformation, the tissue was embedded by scraping all cells on the plate into 3.0 ml of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C.) containing 3 µM imazethapyr from Pursuit® DG. The tissue was broken up and the 3 ml of agarose and tissue were evenly poured onto the surface of a 100x15 mm plate of GN6 (3P). This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured until putative isolates appeared.

#### 8.4.1—Protocol for Isolate Recovery and Regeneration.

Putatively transformed events were isolated off the Pursuit®-containing embedded plates approximately 9 weeks post-transformation by transferring to fresh selection medium of the same concentration in 60x20 mm plates. If

sustained growth was evident after approximately 2-3 weeks, the event was deemed to be resistant and was submitted for molecular analysis.

Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (3P), containing 3 µM imazethapyr from Pursuit® DG, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 µEm<sup>-2</sup> s<sup>-1</sup>) for one week, then higher light (40 µEm<sup>-2</sup> s<sup>-1</sup>) for another week, before being transferred to regeneration medium, 36 (3P), which was identical to 28 (3P) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150x25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L myo-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

From 4 experiments, full plantlets, comprised of a shoot and root, were formed in vitro on the embedded selection plates under dark conditions without undergoing a traditional callus phase. Leaf tissue from nine of these “early regenerators” were submitted for coding region PCR and Plant Transcription Unit (PTU) PCR for the AAD-12 gene and gene cassette, respectively. All had an intact AAD-12 coding region, while 3 did not have a full-length PTU (Table 15). These “early regenerators” were identified as 4101 events to differentiate them from the traditionally-derived events, which were identified as “1283” events. Plants from 19 additional events, obtained via standard selection and regeneration, were sent to the greenhouse, grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T<sub>1</sub> seed. Some of the events appear to be clones of one another due to similar banding patterns following Southern blot, so only 14 unique events were represented. T<sub>0</sub> plants from events were tolerant 70 g/ha imazethapyr. Invader analysis (AHAS gene) indicated insertion complexity ranging from 1 to >10 copies. Thirteen events contained the complete coding region for AAD-12; however, further analysis indicated the complete plant transformation unit had not been incorporated for nine events. None of the compromised 1863 events were advanced beyond the T<sub>1</sub> stage and further characterization utilized the 4101 events.

#### 8.5—Molecular Analysis: Maize Materials and Methods.

8.5.1—Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4° C. for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

8.5.2—Invader assay analysis. The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and MgCl<sub>2</sub> (Third Wave Technologies). An aliquot of 7.5 µl is placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well is overlaid with 15 µl of mineral oil (Sigma). The plates are then incubated at 63° C. for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio.

## US 8,283,522 B2

59

The ratio of known copy standards developed and validated with Southern blot analysis is used to identify the estimated copy of the unknown events.

8.5.3—Polymerase chain reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-12 (v1) PTU are Forward—GAACAGTTAGACATGGTCTAAAGG (SEQ ID NO:8) and Reverse—GCTGCAACACTGATAAATGC-CAACTGG (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 63° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes.

Primers for AAD-12 (v1) Coding Region PCR are Forward—ATGGCTCAGACCACTCTCCAAA (SEQ ID NO:10) and Reverse—AGCTGCATCCATGCCAGGGA (SEQ ID NO:11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

#### 8.5.4—Southern Blot Analysis.

Southern blot analysis is performed with genomic DNA obtained from Qiagen DNeasy kit. A total of 2 µg of genomic leaf DNA or 10 µg of genomic callus DNA is subjected to an overnight digestion using BSM 1 and SWA 1 restriction enzymes to obtain PTU data.

After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85% agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and

60

excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

#### 8.6—Postemergence Herbicide Tolerance in AAD-12 Transformed T<sub>0</sub> Corn.

Four T<sub>0</sub> events were allowed to acclimate in the greenhouse and were grown until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C. under 16 hour light:8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of either Pursuit® (imazethapyr) or 2,4-D Amine 4. Pursuit® was sprayed to demonstrate the function of the selectable marker gene present within the events tested. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. Plants were sprayed with either a lethal dose of imazethapyr (70 g ae/ha) or a rate of 2,4-D DMA salt capable of significant injury to untransformed corn lines (2240 g ae/ha). A lethal dose is defined as the rate that causes >95% injury to the Hi-11 inbred. Hi-11 is the genetic background of the transformants of the present invention.

Several individuals were safened from the herbicides to which the respective genes were to provide resistance. The individual clone '001' from event '001' (a.k.a., 4101(0)-001-001), however, did incur minor injury but recovered by 14 DAT. Three of the four events were moved forward and individuals were crossed with 5XH751 and taken to the next generation. Each herbicide tolerant plant was positive for the presence of the AAD-12 coding region (PCR assay) or the presence of the AHAS gene (Invader assay) for 2,4-D and imazethapyr-tolerant plants, respectively. AAD-12 protein was detected in all 2,4-D tolerant T<sub>0</sub> plants events containing an intact coding region. The copy number of the transgene(s) (AHAS, and by inference AAD-12) varied significantly from 1 to 15 copies. Individual T<sub>0</sub> plants were grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T<sub>1</sub> seed.

TABLE 15

Characterization of T<sub>0</sub> corn plants transformed with AAD-12.

Event	Spray Treatment	% Injury (14 DAT)	AAD-12 ELISA (ppm TSP)	AAD12 PCR (Coding Region)	AAD12 PCR (PTU)	AHAS Copy # (Invader)
4101(0)003.001	2240 g ae/ha 2,4-D	0	146.9	+	+	1
4101(0)003.003	2240 g ae/ha 2,4-D	0	153.5	+	+	1
4101(0)005.001	2240 g ae/ha 2,4-D	0	539.7	+	+	9
4101(0)005.0012	0 g ae/ha 2,4-D	0	562.9	+	+	7
4101(0)001.001	70 g ae/ha imazethapyr	5	170.7	+	+	6
4101(0)002.001	0 g ae/ha imazethapyr	0	105.6	+	-	2
4101(0)002.002	70 g ae/ha imazethapyr	0	105.3	+	-	2
4101(0)003.002	70 g ae/ha imazethapyr	0	0	+	band smaller than expected	15

## US 8,283,522 B2

## 61

8.7—Verification of High 2,4-D Tolerance in T<sub>1</sub> Corn.

T<sub>1</sub> AAD-12 (v1) seed were planted into 3-inch pots containing Metro Mix media and at 2 leaf stage were sprayed with 70 g ae/ha imazethapyr to eliminate nulls. Surviving plants were transplanted to 1-gallon pots containing Metro Mix media and placed in the same growth conditions as before. At V3-V4 stage the plants were sprayed in the track sprayer set to 187 L/ha at either 560 or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to 5XH751×Hi 11 control plants. A grading scale of 0-10 (no injury to extreme auxin injury) was developed to distinguish brace root injury. Brace Root grades were taken on 14DAT to show 2,4-D tolerance. 2,4-D causes brace root malformation, and is a consistent indicator of auxinic herbicide injury in corn. Brace root data (as seen in the table below) demonstrates that 2 of the 3 events tested were robustly tolerant to 2240 g ae/ha 2,4-D DMA. Event “pDAB4101(0)001.001” was apparently unstable; however, the other two events were robustly tolerant to 2,4-D and 2,4-D+imazethapyr or 2,4-D+glyphosate (see Table 16).

TABLE 16

Brace Root injury of AAD-12 (v1) transformed T <sub>1</sub> plants and Untransformed control corn plants.				
Herbicide	Untransformed	AAD-12 (v1)	AAD-12	AAD-12 (v1)
	Control	pDAB4101(0)003.003	(v1)pDAB4101(0)001.001	pDAB4101(0)005.001
Average Brace Root Injury (0-10 Scale)				
0 g ae/ha 2,4-D DMA	0	0	0	0
2240 g ae/ha 2,4-D DMA	9	1	8	0

A scale of 0-10, 10 being the highest, was used for grading the 2,4-D DMA injury. Results are a visual average of four replications per treatment.

## 8.8—AAD-12 (v1) Heritability in Corn.

A progeny test was also conducted on seven AAD-12 (v1) T<sub>1</sub> families that had been crossed with 5XH751. The seeds were planted in three-inch pots as described above. At the 3 leaf stage all plants were sprayed with 70 g ae/ha imazethapyr in the track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Four out of the six lines tested segregated as a single locus, dominant Mendelian

## 62

trait (1R:1S) as determined by Chi square analysis. Surviving plants were subsequently sprayed with 2,4-D and all plants were deemed tolerant to 2,4-D (rates  $\geq$  560 g ae/ha). AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species when reciprocally crossed to a commercial hybrid.

## 8.9—Stacking of AAD-12 (v1) to Increase Herbicide Spectrum

AAD-12 (v1) (pDAB4101) and elite Roundup Ready inbred (BE1146RR) were reciprocally crossed and F<sub>1</sub> seed was collected. The seed from two F<sub>1</sub> lines were planted and treated with 70 g ae/ha imazethapyr at the V2 stage to eliminate nulls. To the surviving plants, reps were separated and either treated with 1120 g ae/ha 2,4-D DMA+70 g ae/ha imazethapyr (to confirm presence of AHAS gene) or 1120 g ae/ha 2,4-D DMA+1680 g ae/ha glyphosate (to confirm the presence of the Round Up Ready gene) in a track sprayer calibrated to 187 L/ha. Plants were graded 3 and 16 DAT.

Spray data showed that AAD-12 (v1) can be conventionally stacked with a glyphosate tolerance gene (such as the Roundup CP4-EPSPS gene) or other herbicide tolerance genes to provide an increased spectrum of herbicides that may be applied safely to corn. Likewise imidazolinone+2,4-D+glyphosate tolerance was observed in F<sub>1</sub> plants and showed no negative phenotype by the molecular or breeding stack combinations of these multiple transgenes.

TABLE 17

Data demonstrating increase herbicide tolerance spectrum resulting from an F <sub>1</sub> stack of AAD-12 (v1) and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF).				
Herbicide	Untransformed Control	2P782 (Roundup Ready Control)	AAD-12 (v1) pDAB4101(0)003.R003.AF	AAD-12 (v1) pDAB4101(0)005.R001.AF
		Average % Injury 16DAT		
0 g ae/ha 2,4-D DMA	0	0	0	0
1120 g ae/ha 2,4-D DMA	21	19	0	0
1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr	100	100	5	1
01120 g ae/ha 2,4-D DMA + 1680 g ae/ha glyphosate	100	71	2	5



## US 8,283,522 B2

63

8.10—Field Tolerance of pDAB4101 Transformed Corn Plants to 2,4-D, Triclopyr and Fluoroxypyr Herbicides.

Field level tolerance trials were conducted on two AAD-12 (v1) pDAB4101 events (4101(0)003.R.003.AF and 4101(0)005.R001.AF) and one Roundup Ready (RR) control hybrid (2P782) at Fowler, Ind. and Wayside, Miss. Seeds were planted with cone planter on 40-inch row spacing at Wayside and 30 inch spacing at Fowler. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluoroxyppyr at 280 g ae/ha and an untreated control. The AAD-12 (v1) events contained the AHAS gene as a selectable marker. The F<sub>2</sub> corn events were segregating so the AAD-12 (v1) plants were treated with imazethapyr at 70 g ae/ha to remove the null plants. Herbicide treatments were applied when corn reached the V6 stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after treatment. Brace root injury ratings were taken at 28DAT on a scale of 0-10 with 0-1 being slight brace root fusing, 1-3 being moderate brace root swelling/wandering and root proliferation, 3-5 being moderate brace root fusing, 5-9 severe brace root fusing and malformation and 10 being total inhibition of brace roots.

AAD-12 (v1) event response to 2,4-D, triclopyr, and fluoroxyppyr at 14 days after treatment are shown in Table 18. Crop injury was most severe at 14 DAT. The RR control corn (2P782) was severely injured (44% at 14 DAT) by 2,4-D at

64

4480 g ae/ha, which is 8 times (8×) the normal field use rate. The AAD-12 (v1) events all demonstrated excellent tolerance to 2,4-D at 14 DAT with 0% injury at the 1, 2 and 4× rates, respectively. The control corn (2P782) was severely injured (31% at 14 DAT) by the 2× rate of triclopyr (840 g ae/ha). AAD-12 (v1) events demonstrated tolerance at 2× rates of triclopyr with an average of 3% injury at 14 DAT across the two events. Fluoroxyppyr at 280 g ae/ha caused 11% visual injury to the wild-type corn at 14 DAT. AAD-12 (v1) events demonstrated increased tolerance with an average of 8% injury at 5 DAT.

Applications of auxinic herbicides to corn in the V6 growth stage can cause malformation of the brace roots. Table 18 shows the severity of the brace root injury caused by 2,4-D, triclopyr, and fluoroxyppyr. Triclopyr at 840 g ae/ha caused the most severe brace root fusing and malformation resulting in an average brace root injury score of 7 in the 2P782 control-type corn. Both AAD-12 (v1) corn events showed no brace root injury from the triclopyr treatment. Brace root injury in 2P782 corn increased with increasing rates of 2,4-D. At 4480 g ae/ha of 2,4-D, the AAD-12 events showed no brace root injury; whereas, severe brace root fusing and malformation was seen in the 2P782 hybrid. Fluoroxyppyr caused only moderate brace root swelling and wandering in the wild-type corn with the AAD-12 (v1) events showing no brace root injury.

This data clearly shows that AAD-12(v1) conveys high level tolerance in corn to 2,4-D, triclopyr and fluoroxyppyr at rates far exceeding those commercially used and that cause non-AAD-12 (v1) corn severe visual and brace root injury.

TABLE 18

Visual Injury of AAD-12 events and wild-type corn following foliar applications of 2,4-D, triclopyr and fluoroxyppyr under field conditions.				
Treatment	Rate (g ae/ha)	% Visual Injury 14DAT		
		AAD-12 4101(0)003.R.003.AF	AAD- 124101(0)005.001.AF	2P782 control
Untreated	0	0	0	0
2,4-D	1120	0	0	9
2,4-D	2240	0	1	20
2,4-D	4480	0	1	34
Fluroxyppyr	280	1	5	11
Triclopyr	840	3	4	31
Dicamba	840	8	8	11

TABLE 19

Brace root injury ratings for AAD-12 and wild-type corn plants in response to 2,4-D, triclopyr and fluoroxyppyr under field conditions.				
Treatment	Rate (g ae/ha)	Brace Root Injury Rating (0-10 scale) 28DAT		
		AAD-12 event 4101(0)003.R.003.AF	AAD-12 event 4101(0)005.001.AF	Wild-type NK603
Untreated	0	0	0	0
2,4-D	1120	0	0	3
2,4-D	2240	0	0	5
2,4-D	4480	0	0	6
Fluroxyppyr	280	0	0	2
Triclopyr	840	0	0	7
Dicamba	840	1	1	1

## US 8,283,522 B2

65

## Example 9

## Protein Detection from Transformed Plants Via Antibody

## 9.1—Extracting AAD-12 (v1) from Plant Leaves.

Approximately 50 to 100 mg of leaf tissue was cut into small pieces (or 4 single-hole-punched leaf discs) and put into 2-ml cluster tubes containing 2 stainless steel BB beads (4.5 mm; Daisy Co., cat. #145462-000). Five hundred microliters of plant extraction buffer (PBS containing 0.05% Tween 20 and 1% Bovine serum albumin) was added to each sample. The tubes were capped and secured in the Geno/Grinder (Model 2000-115, Certiprep, Metuchen, N.J.) and shaken for 6 mM with setting at 1× of 500 rpm. Tubes were centrifuged at 5000×g for 10 mM and supernatant containing the soluble proteins were analyzed for AAD-12 (v1) using Western Blots and ELISA.

## 9.2—Enzyme Linked Immuno-Sorbent Assay (ELISA).

The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-12 antibody (0.5 µg/ml) was coated on 96-well microtiter well and incubated at 4° C. for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 µL standard AAD-12 of known concentrations or plant extracts from different samples were incubated in the wells. For standard curve, purified AAD-12 was diluted 2-fold serially from 52 to 0.813 ng/ml in triplicates. Plant extracts were diluted 5, 10, 20, and 40-fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-12 antibody-HRP conjugate (0.5 µg/ml) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce, Rockford, Ill.), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 µL 0.4N H<sub>2</sub>SO<sub>4</sub>. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-12 (v1) in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

For comparison, each sample was normalized with its fresh weight and percent expression was calculated.

## 9.3—Western Blotting Analysis.

Plant extracts or AAD-12 standards (5 and 0.5 µg/ml) were incubated with Laemmli sample buffer at 95° C. for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-12 (v1) protein was detected by anti-AAD-12 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham, N.J.).

## Example 10

## Tobacco Transformation

Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch et al., 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. KY160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige

66

and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C. and leaves collected sterily for use in the transformation protocol. Pieces of approximately one square centimeter were sterily cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB3278, aka pDAS1580, AAD-12 (v1)+PAT), grown overnight in a flask on a shaker set at 250 rpm at 28° C., were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Fla.) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C. in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ 11 vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C. greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C., 16 hour day, 8 hour night, minimum natural+supplemental light=500 µE/m<sup>2</sup> s<sup>-1</sup>).

Prior to propagation, T<sub>0</sub> plants were sampled for DNA analysis to determine the insert copy number. The PAT gene which was molecularly linked to AAD-12 (v1) was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µL.

The DNA samples were diluted to 9 ng/µL and then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgCl<sub>2</sub> (Third Wave Technologies). An aliquot of 7.5 µL was placed in each well of the Invader assay plate followed by an aliquot of 7.5 µL of controls, standards, and 20 ng/µL diluted unknown samples. Each well was overlaid with 15 µL of mineral oil (Sigma). The plates were then incubated at 63° C. for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy stan-

## US 8,283,522 B2

67

dards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

All events were also assayed for the presence of the AAD-12 (v1) gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit. Primers for the Plant Transcription Unit (PTU) PCR AAD-12 were (SdpacodF: ATGGCTCA TGCTGC-CCTCAGCC) (SEQ ID NO:12) and (SdpacodR: CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:13). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 64° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products

68

pyr was likewise applied at 35, 140, or 560 g ae/ha. Triclopyr was applied at 70, 280, or 1120 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 560 g ae/ha 2,4-D or less. Some events were uninjured at 2,4-D applied at 2240 g ae/ha (equivalent to 4× field rate). On the whole, AAD-12 (v1) events were more sensitive to fluoroxyppy, followed by triclopyr, and least affected by 2,4-D. The quality of the events with respect to magnitude of resistance was discerned using T<sub>0</sub> plant responses to 560 g ae/ha fluoroxyppy. Events were categorized into “low” (>40% injury 14 DAT), “medium” (20-40% injury), “high” (<20% injury). Some events were inconsistent in response among replicates and were deemed “variable.”

TABLE 20

Tobacco T0 events transformed with pDAS1580 (AAD-12 (v1) + PAT)						
#	Plant ID	Copy #	PTU PCR AAD-12	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance <sup>@</sup>
1	1580[1]-001	6	+			Not tested
2	1580[1]-002	8	+			Not tested
3	1580[1]-003	10	+			Not tested
4	1580[1]-004	1	+	*	*	High
5	1580[1]-005	2	+	*		Variable
6	1580[1]-006	6	+			Not tested
7	1580[1]-007	4	+			Not tested
8	1580[1]-008	3	+			Variable
9	1580[1]-009	4	+			Not tested
10	1580[1]-010	8	+			Not tested
11	1580[1]-011	3	+			High
12	1580[1]-012	12	+			Not tested
13	1580[1]-013	13	+			Not tested
14	1580[1]-014	4	+			Not tested
15	1580[1]-015	2	+	*		High
16	1580[1]-016	1 ?	+	*	*	High
17	1580[1]-017	3	+			High
18	1580[1]-018	1	+	*	*	Variable
19	1580[1]-019	1	+	*	*	Variable
20	1580[1]-020	1	+	*	*	Not tested
21	1580[1]-021	1	+	*	*	Not tested
22	1580[1]-022	3	+			Variable
23	1580[1]-023	1	+	*	*	Variable
24	1580[1]-024	1	+	*	*	Variable
25	1580[1]-025	5	+			Not tested
26	1580[1]-026	3	+			Variable
27	1580[1]-027	3	+			Low
28	1580[1]-028	4	+			Not tested
29	1580[1]-029	3	+			Variable
30	1580[1]-030	1	+	*	*	High
31	1580[1]-031	1	+	*	*	High
32	1580[1]-032	2	+	*		High

<sup>@</sup>Distinguishing herbicide tolerance performance of events required assessment of relative tolerance when treated with 560 g ae/ha fluoroxyppy where tolerance was variable across events.

were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 18 PCR positive events with 1-3 copies of PAT gene (and presumably AAD-12 (v1) since these genes are physically linked) were regenerated and moved to the greenhouse.

#### 10.1 Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T<sub>0</sub> Tobacco

T<sub>0</sub> plants from each of the 19 events were challenged with a wide range of 2,4-D, triclopyr, or fluoroxyppy sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 140, 560, or 2240 g ae/ha to representative clones from each event mixed in deionized water. Fluoroxy-

#### 10.2 Verification of High 2,4-D Tolerance in T<sub>1</sub> Tobacco.

Two to four T<sub>0</sub> individuals surviving high rates of 2,4-D and fluoroxyppy were saved from each event and allowed to self fertilize in the greenhouse to give rise to T<sub>1</sub> seed. The T<sub>1</sub> seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 7.4), followed by selective removal of untransformed nulls in this segregating population with 560 g ai/ha glufosinate (PAT gene selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided high levels of resistance to 2,4-D in the T<sub>0</sub> generation. Improved consistency of response is anticipated in T<sub>1</sub> plants not having come directly from tissue culture. These plants were compared against wildtype KY160 tobacco. All plants were sprayed with a track sprayer set at



## US 8,283,522 B2

69

187 L/ha. The plants were sprayed from a range of 140-2240 g ae/ha 2,4-D dimethylamine salt (DMA), 70-1120 g ae/ha triclopyr or 35-560 g ae/ha fluoroxyppr. All applications were formulated in water. Each treatment was replicated 2-4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T<sub>1</sub> generation is segregating, so some variable response is expected due to difference in zygosity.

No injury was observed at 4× field rate (2240 g ae/ha) for 2,4-D or below. Some injury was observed with triclopyr treatments in one event line, but the greatest injury was observed with fluoroxyppr. The fluoroxyppr injury was short-lived and new growth on one event was nearly indistinguishable from the untreated control by 14 DAT (Table 21). It is important to note that untransformed tobacco is exceedingly sensitive to fluoroxyppr. These results indicated commercial level 2,4-D tolerance can be provided by AAD-12 (v1), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to the pyridyloxyacetic acid herbicides, triclopyr and fluoroxyppr. Having the ability to prescribe treatments in an herbicide tolerant crop protected by AAD-12 with various active ingredients having varying spectra of weed control is extremely useful to growers.

TABLE 21

Assessment of cross tolerance of AAD-12 (v1) T <sub>1</sub> tobacco plants' response to various phenoxy and pyridyloxy auxin herbicides.			
Herbicide	KY160-Wildtype Average % Injury of Replicates	1580(1)-004 (high tolerance in T <sub>0</sub> generation)	1580(1)-018 (high tolerance in T <sub>0</sub> generation)
		14 DAT	
140 g ae/ha 2,4-D DMA	45	0	0
560 g ae/ha 2,4-D DMA	60	0	0
2240 g ae/ha 2,4-D DMA	73	0	0
70 g ae/ha triclopyr	40	0	5
280 g ae/ha triclopyr	65	0	5
1120 g ae/ha triclopyr	80	0	8
35 g ae/ha fluoroxyppr	85	0	8
140 g ae/ha fluoroxyppr	93	0	10
560 g ae/ha fluoroxyppr	100	3	18

## 10.3 AAD-12 (v1) Heritability in Tobacco

A 100 plant progeny test was also conducted on seven T<sub>1</sub> lines of AAD-12 (v1) lines. The seeds were stratified, sown, and transplanted with respect to the procedure above with the exception that null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Five out of the seven lines

70

tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species.

## 10.4—Field Tolerance of pDAS1580 Tobacco Plants to 2,4-D, Dichloprop, Triclopyr and Fluoroxyppr Herbicides.

Field level tolerance trials were conducted on three AAD-12 (v1) lines (events pDAS1580-[1]-018.001, pDAS1580-[1]-004.001 and pDAS1580-[1]-020.016) and one wild-type line (KY160) at field stations in Indiana and Miss. Tobacco transplants were grown in the greenhouse by planting T<sub>1</sub> seed in 72 well transplant flats (Hummert International) containing Metro 360 media according to growing conditions indicated above. The null plants were selectively removed by Liberty selection as previously described. The transplant plants were transported to the field stations and planted at either 14 or 24 inches apart using industrial vegetable planters. Drip irrigation at the Mississippi site and overhead irrigation at the Indiana site were used to keep plants growing vigorously.

The experimental design was a split plot design with 4 replications. The main plot was herbicide treatment and the sub-plot was tobacco line. The herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluoroxyppr at 280 g ae/ha and an untreated control. Plots were one row by 25-30 ft. Herbicide treatments were applied 3-4 weeks after transplanting using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual rating of injury, growth inhibition, and epinasty were taken at 7, 14 and 21 days after treatment.

AAD-12 (v1) event response to 2,4-D, triclopyr, and fluoroxyppr are shown in Table 22. The non-transformed tobacco line was severely injured (63% at 14 DAT) by 2,4-D at 560 g ae/ha which is considered the 1× field application rate. The AAD-12 (v1) lines all demonstrated excellent tolerance to 2,4-D at 14 DAT with average injury of 1, 4, and 4% injury observed at the 2, 4 and 8× rates, respectively. The non-transformed tobacco line was severely injured (53% at 14 DAT) by the 2× rate of triclopyr (840 g ae/ha); whereas, AAD-12 (v1) lines demonstrated tolerance with an average of 5% injury at 14 DAT across the three lines. Fluoroxyppr at 280 g ae/ha caused severe injury (99%) to the non-transformed line at 14 DAT. AAD-12 (v1) lines demonstrated increased tolerance with an average of 11% injury at 14 DAT.

These results indicate that AAD-12 (v1) transformed event lines displayed a high level of tolerance to 2,4-D, triclopyr and fluoroxyppr at multiples of commercial use rates that were lethal or caused severe epinastic malformations to non-transformed tobacco under representative field conditions.

TABLE 22

AAD-12 (v1) tobacco plants response to 2,4-D, triclopyr, and fluoroxyppr under field conditions.					
Average % Injury across locations at 14 DAT					
Herbicide Treatment		Wild	pDAS1580-	pDAS1580-	pDAS1580-
Active Ingredient	Rate	type	[1]-004.001	[1]-020.016	[1]-018.001
2,4-D	280 GM AE/HA	48	0	0	0
2,4-D	560 GM AE/HA	63	0	0	2
2,4-D	1120 GM AE/HA	78	1	1	2
2,4-D	2240 GM AE/HA	87	4	4	4
2,4-D	4480 GM AE/HA	92	4	4	4
Triclopyr	840 GM AE/HA	53	5	5	4
Fluroxyppr	280 GM AE/HA	99	11	11	12

## US 8,283,522 B2

71

## 10.5 AAD-12 (v1) Protection Against Elevated 2,4-D Rates

Results showing AAD-12 (v1) protection against elevated rates of 2,4-D DMA in the greenhouse are shown in Table 23. T<sub>1</sub> AAD-12 (v1) plants from an event segregating 3R:1S when selected with 560 g ai/ha Liberty using the same protocol as previously described. T<sub>1</sub> AAD-1 (v3) seed was also planted for transformed tobacco controls (see PCT/US2005/014737). Untransformed KY160 was served as the sensitive control. Plants were sprayed using a track sprayer set to 187 L/ha at 140, 560, 2240, 8960, and 35840 g ae/ha 2,4-D DMA and rated 3 and 14 DAT.

72

respectively). The complementary nature of herbicide cross resistance patterns allows convenient use of these two genes as complementary and stackable field-selectable markers. In crops where tolerance with a single gene may be marginal, one skilled in the art recognizes that one can increase tolerance by stacking a second tolerance gene for the same herbicide. Such can be done using the same gene with the same or different promoters; however, as observed here, stacking and tracking two complementary traits can be facilitated by the distinguishing cross protection to phenoxypyroic acids [from AAD-1 (v3)] or pyridyloxyacetic acids [AAD-12 (v1)]

TABLE 24

Comparison of auxinic herbicide cross tolerance of AAD-12 (v1) (pDAS1580) and AAD-1 (v3) (pDAB721) T <sub>2</sub> plants compared to AAD-12 × AAD-1 F <sub>1</sub> cross and to wildtype.				
Treatment	KY160 wildtype control	AAD-12 (v1) (pDAS1580)	AAD-1(v3) (pDAB721)	AAD-12 (v1) × AAD (v3) F <sub>1</sub>
Average % injury 14 DAT				
560 g ae/ha 2,4-D	63	0	0	0
1120 g ae/ha 2,4-D	80	0	4	0
2240 g ae/ha 2,4-D	90	0	9	0
280 g ae/ha R-dichlorprop	25	15	0	0
560 g ae/ha R-dichlorprop	60	50	0	0
1120 g ae/ha R-dichlorprop	80	70	3	0
70 g ae/ha fluroxypyr	40	0	40	0
140 g ae/ha fluroxypyr	65	0	60	0
280 g ae/ha fluroxypyr	75	3	75	3

AAD-12 (v1) and AAD-1 (v3) both effectively protected tobacco against 2,4-D injury at doses up to 4× commercial use rates. AAD-12 (v1), however, clearly demonstrated a marked advantage over AAD-1 (v3) by protecting up to 64× the standard field rates.

## Example 11

## Soybean Transformation

Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgett et al., 1995), amino acid modification (Falco et al., 1995), and insect resistance (Parrott et al., 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe et al., 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchey et al., 1988) or zygotic embryos (Chee et al., 1989) have been reported.

Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee et al., 1989; McCabe et al., 1988), cotyledon (Hinchey et al., 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh et al., 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L

TABLE 23

Results demonstrating protection provided by AAD-12 (v1) and AAD-1 (v3) against elevated rates of 2,4-D.			
Treatment	KY160 control	AAD-1(v3)	AAD-12 (v1)
Average % injury 14 DAT			
2240 g ae/ha 2,4-D	95	4	0
8960 g ae/ha 2,4-D	99	9	0
35840 g ae/ha 2,4-D	100	32	4

## 10.6 Stacking of AAD-12 to Increase Herbicide Spectrum

Homozygous AAD-12 (v1) (pDAS1580) and AAD-1 (v3) (pDAB721) plants (see PCT/US2005/014737 for the latter) were both reciprocally crossed and F<sub>1</sub> seed was collected. The F<sub>1</sub> seed from two reciprocal crosses of each gene were stratified and treated 4 reps of each cross were treated under the same spray regime as used for the other testing with one of the following treatments: 70, 140, 280 g ae/ha fluroxypyr (selective for the AAD-12 (v1) gene); 280, 560, 1120 g ae/ha R-dichlorprop (selective for the AAD-1 (v3) gene); or 560, 1120, 2240 g ae/ha 2,4-D DMA (to confirm 2,4-D tolerance). Homozygous T<sub>2</sub> plants of each gene were also planted for use as controls. Plants were graded at 3 and 14 DAT. Spray results are shown in Table 24.

The results confirm that AAD-12 (v1) can be successfully stacked with AAD-1 (v3), thus increasing the spectrum herbicides that may be applied to the crop of interest (phenoxypyracetic acids+phenoxypyroic acids vs phenoxypyracetic acids+pyridyloxyacetic acids for AAD-1 and AAD-12,

concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the AAD-12 (v1) gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using AAD-12 (v1).

#### 11.1 Gateway Cloning of Binary Constructs

The AAD-12 (v1) coding sequence was cloned into five different Gateway Donor vectors containing different plant promoters. The resulting AAD-12 (v1) plant expression cassettes were subsequently cloned into a Gateway Destination Binary vector via the LR Clonase reaction (Invitrogen Corporation, Carlsbad Calif., Cat #11791-019).

An NcoI-SacI fragment containing the AAD-12 (v1) coding sequence was digested from DASPICO12 and ligated into corresponding NcoI-SacI restriction sites within the following Gateway Donor vectors: pDAB3912 (attL1//CsVMV promoter//AtuORF23 3'UTR//attL2); pDAB3916 (attL1//AtUbi10 promoter//AtuORF23 3'UTR//attL2); pDAB4458 (attL1//AtUbi3 promoter//AtuORF23 3'UTR//attL2); pDAB4459 (attL1//ZmUbi1 promoter//AtuORF23 3'UTR//attL2); and pDAB4460 (attL1//AtAct2 promoter//AtuORF23 3'UTR//attL2). The resulting constructs containing the following plant expression cassettes were designated: pDAB4463 (attL1//CsVMV promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4467 (attL1//AtUbi10 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4471 (attL1//AtUbi3 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4475 (attL1//ZmUbi1 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); and pDAB4479 (attL1//AtAct2 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2). These constructs were confirmed via restriction enzyme digestion and sequencing.

The plant expression cassettes were recombined into the Gateway Destination Binary vector pDAB4484 (RB7 MARv3//attR1-ccdB-chloramphenicol resistance-attR2//CsVMV promoter//PATv6//AtuORF1 3'UTR) via the Gateway LR Clonase reaction. Gateway Technology uses lambda phage-based site-specific recombination instead of restriction endonuclease and ligase to insert a gene of interest into an expression vector. Invitrogen Corporation, Gateway Technology: A Universal Technology to Clone DNA Sequences for Functional Analysis and Expression in multiple Systems, Technical Manual, Catalog #'s 12535-019 and 12535-027, Gateway Technology Version E, Sep. 22, 2003, #25-022. The DNA recombination sequences (attL, and attR,) and the LR Clonase enzyme mixture allows any DNA fragment flanked by a recombination site to be transferred into any vector containing a corresponding site. The attL1 site of the donor vector corresponds with attR1 of the binary vector. Likewise, the attL2 site of the donor vector corresponds with attR2 of the binary vector. Using the Gateway Technology the plant expression cassette (from the donor vector) which is flanked by the attL sites can be recombined into the attR sites of the binary vector. The resulting constructs containing the following plant expression cassettes were labeled as: pDAB4464 (RB7 MARv3//CsVMV promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6 AtuORF1 3'UTR); pDAB4468 (RB7 MARv3//AtUbi10 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4472 (RB7 MARv3//AtUbi3 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4476 (RB7 MARv3//ZmUbi1 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6 AtuORF1 3'UTR); and pDAB4480 (RB7 MARv3//AtAct2 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//

PATv6//AtuORF1 3'UTR) (see Table 8). These constructs were confirmed via restriction enzyme digestion and sequencing.

#### 11.2 Transformation Method 1: Cotyledonary Node Transformation of Soybean Mediated by *Agrobacterium tumefaciens*.

The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee et al., 1988) and shoot multiplication from apical meristems (McCabe et al., 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee et al., 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente et al., 2000; Olhoft et al., 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on micro-projectile bombardment (McCabe et al., 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell et al., 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This de novo meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee et al., 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang et al., 1999; Zeng et al., 2004).

11.2.1—Plant transformation production of AAD-12 (v1) tolerant phenotypes. Seed derived explants of "Maverick" and the *Agrobacterium* mediated cot-node transformation protocol was used to produces AAD-12 (v1) transgenic plants.

#### 11.2.2—*Agrobacterium* Preparation and Inoculation

*Agrobacterium* strain EHA101 (Hood et al. 1986), carrying each of five binary pDAB vectors (Table 8) was used to initiate transformation. Each binary vector contains the AAD-12 (v1) gene and a plant-selectable gene (PAT) cassette within the T-DNA region. Each gene is driven by the promoters listed in Table 8 and these plasmids were mobilized into the EHA101 strain of *Agrobacterium* by electroporation. The selected colonies were then analyzed for the integration of genes before the *Agrobacterium* treatment of the soybean explants. Maverick seeds were used in all transformation experiments and the seeds were obtained from University of Missouri, Columbia, Mo.

*Agrobacterium*-mediated transformation of soybean (*Glycine max*) using the PAT gene as a selectable marker coupled with the herbicide glufosinate as a selective agent was carried out followed a modified procedure of Zeng et al. (2004). The seeds were germinated on B5 basal medium (Gamborg et al. 1968) solidified with 3 g/L Phytagel (Sigma-Aldrich, St. Louis, Mo.); added 1-cysteine to the co-cultivation medium at 400 mg/L and co-cultivation lasted 5 days (Olhoft and Somers 2001); shoot initiation, shoot elongation, and rooting media were supplemented with 50 mg/L cefotaxime, 50 mg/L

US 8,283,522 B2

75

timentin, 50 mg/L vancomycin, and solidified with 3 g/L Phytigel. Selected shoots were then transferred to the rooting medium. The optimal selection scheme was the use of glufosinate at 8 mg/L across the first and second shoot initiation stages in the medium and 3-4 mg/L during shoot elongation in the medium.

Prior to transferring elongated shoots (3-5 cm) to rooting medium, the excised end of the internodes were dipped in 1 mg/L indole 3-butyric acid for 1-3 min to promote rooting (Khan et al. 1994). The shoots struck roots in 25×100 mm glass culture tubes containing rooting medium and then they were transferred to soil mix for acclimatization of plantlets in Metro-mix 200 (Hummert International, Earth City, Mo.) in open Magenta boxes in Conviron. Glufosinate, the active ingredient of Liberty herbicide (Bayer Crop Science), was used for selection during shoot initiation and elongation. The rooted plantlets were acclimated in open Magenta boxes for several weeks before they were screened and transferred to the greenhouse for further acclimation and establishment.

#### 11.2.3—Assay of Putatively Transformed Plantlets, and Analyses Established T<sub>0</sub> Plants in the Greenhouse.

The terminal leaflets of selected leaves of these plantlets were leaf painted with 50 mg/L of glufosinate twice with a week interval to observe the results to screen for putative transformants. The screened plantlets were then transferred to the greenhouse and after acclimation the leaves were painted with glufosinate again to confirm the tolerance status of these plantlets in the GH and deemed to be putative transformants.

Plants that are transferred to the greenhouse can be assayed for the presence of an active PAT gene further with a non-destructive manner by painting a section of leaf of the T<sub>0</sub> primary transformant, or progeny thereof, with a glufosinate solution [0.05-2% v/v Liberty Herbicide, preferably 0.25-1.0% (v/v),=500-2000 ppm glufosinate, Bayer Crop Science]. Depending on the concentration used, assessment for glufosinate injury can be made 1-7 days after treatment. Plants can also be tested for 2,4-D tolerance in a non-destructive manner by selective application of a 2,4-D solution in water (0.25-1% v/v commercial 2,4-D dimethylamine salt formulation, preferably 0.5% v/v=2280 ppm 2,4-D ae) to the terminal leaflet of the newly expanding trifoliolate one or two, preferably two, nodes below the youngest emerging trifoliolate. This assay allows assessment of 2,4-D sensitive plants 6 hours to several days after application by assessment of leaf flipping or rotation >90 degrees from the plane of the adjacent

76

leaflets. Plants tolerant to 2,4-D will not respond to 2,4-D. T<sub>0</sub> plants will be allowed to self fertilize in the greenhouse to give rise to T<sub>1</sub> seed. T<sub>1</sub> plants (and to the extent enough T<sub>0</sub> plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by AAD-12 (v1) and PAT genes in transgenic soybean. Rates of 2,4-D used on T<sub>0</sub> plants will typically comprise one or two selective rates in the range of 100-1120 g ae/ha using a track sprayer as previously described. T<sub>1</sub> plants will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T<sub>0</sub> and T<sub>1</sub> plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Glyphosate resistance (in plants transformed with constructs that contain EPSPS) or another glyphosate tolerance gene can be assessed in the T<sub>1</sub> generation by postemergence applications of glyphosate with a dose range from 280-2240 g ae/ha glyphosate. Analysis of protein expression will occur as described in below. Individual T<sub>0</sub> plants were assessed for the presence of the coding region of the gene of interest (AAD-12 (v1) or PAT v6) and copy number. Determination of the inheritance of AAD-12 (v1) will be made using T<sub>1</sub> and T<sub>2</sub> progeny segregation with respect to herbicide tolerance as described in previous examples.

A subset of the initial transformants were assessed in the T<sub>0</sub> generation according to the methods above. Any plant confirmed as having the AAD-12 (v1) coding region, regardless of the promoter driving the gene did not respond to the 2,4-D leaf painting whereas wildtype Maverick soybeans did (Table Sec 11.2.3). PAT-only transformed plants responded the same at wildtype plants to leaf paint applications of 2,4-D

2,4-D was applied to a subset of the plants that were of similar size to the wildtype control plants with either 560 or 1120 g ae 2,4-D. All AAD-12 (v1)-containing plants were clearly resistant to the herbicide application versus the wildtype Maverick soybeans. A slight level of injury (2 DAT) was observed for two AAD-12 (v1) plants, however, injury was temporary and no injury was observed 7 DAT. Wildtype control plants were severely injured 7-14 DAT at 560 g ae/ha 2,4-D and killed at 1120 g ae/ha. These data are consistent with the fact that AAD-12 (v1) can impart high tolerance (>2× field rates) to a sensitive crop like soybeans. The screened plants were then sampled for molecular and biochemical analyses for the confirmation of the AAD12 (v1) genes integration, copy number, and their gene expression levels as described below and reported in Table 25.

TABLE 25

T<sub>0</sub> soybean response to 2,4-D leaf paint and 2,4-D spray application.

				Leaf flip assay 2,4-D @ (18 HAT)										
				NODE LEAF	Node		Spray POST over the top with 2,4-D	Stage at appl (# nodes)	ELISA]	Southern Copy	PCR coding	% injury 2	% injury 7	% injury 14
Construct														
(pDAB#)	Gene	Promoter	Event	PAINTED	N-1	N-2	(g ae/ha)		(ng/mL)	number	region	DAT	DAT	DAT
4464	AAD-12	CsVMV	D-1-14	N-1	0		0	>10	5246.83	2	+	X	X	0
4464	AAD-12	CsVMV	D-2-9	N-2		0	0	>10	204.27	1	+	X	X	0
4468	AAD-12	AtUbi10	D-3-7	N-2		0	0	>10	4.65	1	+	0	0	0
4468	AAD-12	AtUbi10	D-4-11B	N-2		0	0	8	1452.84	2	+	0	0	0
4468	AAD-12	AtUbi10	D-4-16	N-2		0	0	>10	653.21	2	+	X	X	0
4480	AAD-12	AtAct2	D-9-1	N-2		0	0	>10	248.33	3 or 4	+	X	X	0
4464	AAD-12	CsVMV	D-2-14	N-2		0	560	7	4917.43	2	+	0	0	0
4468	AAD-12	AtUbi10	D-3-5	N-2		0	560	8	365.75	1	+	0	0	0
4468	AAD-12	AtUbi10	D-3-6	N-1	0		560	5	714.79	3	+	0	0	0
4472	AAD-12	AtUbi3	D-5-2	N-1	0		560	6	0.58	1	+	5	0	0
4468	AAD-12	AtUbi10	D-3-9	N-2		0	1120	6	2657.26	3	+	0	0	0



## US 8,283,522 B2

77

78

TABLE 25-continued

T <sub>0</sub> soybean response to 2,4-D leaf paint and 2,4-D spray application.														
Leaf flip assay 2,4-D @ (18 HAT)														
Construct				NODE LEAF	Node		Spray POST over the top with 2,4-D	Stage at appl (#	ELISA]	Southern Copy	PCR coding	% injury 2	% injury 7	% injury 14
(pDAB#)	Gene	Promoter	Event	PAINTED	N-1	N-2	(g ae/ha)	nodes)	(ng/mL)	number	region	DAT	DAT	DAT
4468	AAD-12	AtUbi10	D-4-17	N-2		0	1120	7	286.14	5	+	5	0	0
4499	PAT	CsVMV	D-2-3	N-2		1	0	>10	2.36	5	+	X	X	0
Maverick	WT		WT-10	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-2	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-3	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-4	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-5	NT			560	4	ND	ND	ND	50	60	60
Maverick	WT		WT-6	NT			560	4	ND	ND	ND	70	90	80
Maverick	WT		WT-7	NT			560	4	ND	ND	ND	70	80	80
Maverick	WT		WT-10	NT			1120	4	ND	ND	ND	70	90	100
Maverick	WT		WT-8	NT			1120	4	ND	ND	ND	70	95	100
Maverick	WT		WT-9	NT			1120	4	ND	ND	ND	70	95	100

1 = Flip

0 = No Flip

ND = Not determined

## 11.2.4—Molecular Analyses: Soybean

11.2.4.1—Tissue harvesting DNA isolation and quantification. Fresh tissue is placed into tubes and lyophilized at 4° C. for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μL.

11.2.4.2—Polymerase chain reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-12 (v1) PTU are (Forward—ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:8) and (Reverse—CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 63° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. Primers for Coding Region PCR AAD-12 (v1) are (Forward—ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:10) and (Reverse—CGGGC AGGCCTAACTCCAC-CAA) (SEQ ID NO:11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

11.2.4.3—Southern blot analysis. Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 10 μg of genomic DNA is subjected to an overnight digestion to obtain integration data. After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run

25

on a large 0.85% agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

## 11.2.5—Biochemical Analyses: Soybean

11.2.5.1—Tissue Sampling and Extracting AAD-12 (v1) protein from soybean leaves. Approximately 50 to 100 mg of leaf tissue was sampled from the N-2 leaves that were 2,4-D leaf painted, but after 1 DAT. The terminal N-2 leaflet was removed and either cut into small pieces or 2-single-hole-punched leaf discs (~0.5 cm in diameter) and were frozen on dry ice instantly. Further protein analysis (ELISA and Western analysis) was completed according to methods described in Example 9.

11.2.6—T<sub>1</sub> Progeny evaluation. T<sub>0</sub> plants will be allowed to self fertilize to derive T<sub>1</sub> families. Progeny testing (segre-

65

US 8,283,522 B2

79

gation analysis) will be assayed using glufosinate at 560 g ai/ha as the selection agent applied at the V1-V2 growth stage. Surviving plants will be further assayed for 2,4-D tolerance at one or more growth stages from V2-V6. Seed will be produced through self fertilization to allow broader herbicide testing on the transgenic soybean.

AAD-12 (v1) transgenic Maverick soybean plants have been generated through *Agrobacterium*-mediated cot-node transformation system. The T<sub>0</sub> plants obtained tolerated up to 2× levels of 2,4-D field applications and developed fertile seeds. The frequency of fertile transgenic soybean plants was up to 5.9%. The integration of the AAD1-12 (v1) gene into the soybean genome was confirmed by Southern blot analysis. This analysis indicated that most of the transgenic plants contained a low copy number. The plants screened with AAD-12 (v1) antibodies showed positive for ELISA and the appropriate band in Western analysis.

11.3 Transformation Method 2: Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue.

Culture of embryogenic soybean callus tissue and subsequent beaming can be accomplished as described in U.S. Pat. No. 6,809,232 (Held et al.) to create transformants using one or more constructs in Table 8.

11.4 Transformation Method 3. Biolistic Bombardment of Soybean

This can be accomplished using mature seed derived embryonic axes meristem (McCabe et al. (1988)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

11.5 Transformation Method 4. Whiskers Mediated Transformation.

Whisker preparation and whisker transformation can anticipated according to methods described previously by Terakawa et al. (2005)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

Maverick seeds were surface-sterilized in 70% ethanol for 1 min followed by immersion in 1% sodium hypochlorite for 20 min. and then rinsed three times in sterile distilled water. The seeds were soaked in distilled water for 18-20 h. The embryonic axes were excised from seeds, and the apical meristems were exposed by removing the primary leaves. The embryonic axes were positioned in the bombardment medium [BM: MS (Murashige and Skoog 1962) basal salts medium, 3% sucrose and 0.8% phytagel Sigma, pH 5.7] with the apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium.

11.6 Transformation Method 5. Particle bombardment-mediated transformation for embryogenic callus tissue can be optimized for according to previous methods (Khalafalla et al., 2005; El-Shemy et al., 2004, 2006). Regenerated plants can also be assessed according to Example 11.2.

#### Example 12

##### AAD-12 (v1) in Cotton

###### 12.1—Cotton Transformation Protocol.

Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% com-

80

mercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (Table 26) in Magenta GA-7 vessels and maintained under high light intensity of 40-60  $\mu\text{E}/\text{m}^2$ , with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

Cotyledon segments (~5 mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (Table 26) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (Table 26) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (Table 26). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

*Agrobacterium* preparation. Inoculate 35 ml of Y media (Table 26) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C., while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (Table 26) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10<sup>8</sup> colony forming units per ml with a total volume of 40 ml.

After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. In a side-by-side comparison, MG media can be supplemented with dichlorprop (added to the media at a concentration of 0.01 and 0.05 mg/L) to supplement for the degradation of the 2,4-D, since dichlorprop is not a substrate for to the AAD-12 enzyme, however dichlorprop is more active on cotton than 2,4-D. In a separate comparison, segments which were plated on MG media containing no growth regulator compared to standard MG media, showed reduced callusing, but there still is callus growth. Callus is then transferred to CG-media (Table 26), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 26) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color. Cotton can take time to regenerate and form embryos, one of the ways to speed up this process is to stress the tissue. Dessication is a common way to accomplish this, via changes in the microenvironment of the tissue and plate, by using less culture media and/or adopting various modes of plate enclosure (taping versus parafilm).

Larger, well-developed embryos are isolated and transferred to DK media (Table 26) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.



## US 8,283,522 B2

81

82

TABLE 26

Media for Cotton Transformation								
Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1 mM)		1 ml	1 ml	1 ml	4.6 ml		0.5 ml	
2,4-D (1 mM)		1 ml	1 ml	1 ml				
Agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10 mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

## 12.2—Cell Transformation.

Several experiments were initiated in which cotyledon segments were treated with *Agrobacterium* containing pDAB724. Over 2000 of the resulting segments were treated using various auxin options for the proliferation of pDAB724 cotton callus, either: 0.1 or 0.5 mg/L R-dichlorprop, standard 2,4-D concentration and no auxin treatment. The callus was selected on glufosinate-ammonium, due to the inclusion of the PAT gene in the construct. Callus line analysis in the form of PCR and Invader will be used to determine if and to be sure the gene was present at the callus stage; then callus lines that are embryogenic will be sent for Western analysis, essentially as described in section 11.2.3. Embryogenic cotton callus was stressed using dessication techniques to improve the quality and quantity of the tissue recovered.

Almost 200 callus events have been screened for intact PTU and expression using Western analysis for the AAD-12 (v1) gene. Below is a subset of the data for some of the cotton callus that has been tested.

Table 26.b

Construct	Line Number	AAD-12 PTU	AAD-12 Invader	AAD-12 ng/ml
PDAB724	1	+	+	79.89
PDAB724	2	+	+	17.34
PDAB724	3	+	+	544.80
PDAB724	4	+	+	32.63
PDAB724	5	+	+	82.77
PDAB724	83	+	+	795.50
PDAB724	84	+	+	613.35
PDAB724	85	+	+	1077.75
PDAB724	86	+	+	437.74
PDAB724	87	+	+	286.51
PDAB724	88	+	+	517.59
PDAB724	89	+	+	1250.70

## 12.3—Plant Regeneration.

AAD-12 (v1) cotton lines that have produced plants according to the above protocol will be sent to the greenhouse. To demonstrate the AAD-12 (v1) gene provides resistance to 2,4-D in cotton, both the AAD-12 (v1) cotton plant and wild-type cotton plants will be sprayed with a track sprayer delivering 560 g ae/ha 2,4-D at a spray volume of 187

L/ha. The plants will be evaluated at 3 and 14 days after treatment. Plants surviving a selective rate of 2,4-D will be self pollinated to create T<sub>1</sub> seed or outcrossed with an elite cotton line to produce F<sub>1</sub> seed. The subsequent seed produced will be planted and evaluated for herbicide resistance as previously described. AAD-12 (v1) events can be combined with other desired HT or IR traits as described in experiments 18, 19, 22, and 23.

## Example 13

*Agrobacterium* Transformation of Other Crops

In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated trans-formation of rye, see, e.g., Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, see, e.g., Hinchee et al., 1988. For *Agrobacterium*-mediated transformation of sorghum, see, e.g., Zhao et al., 2000. For *Agrobacterium*-mediated transformation of barley, see, e.g., Tingay et al., 1997. For *Agrobacterium*-mediated transformation of wheat, see, e.g., Cheng et al., 1997. For *Agrobacterium*-mediated transformation of rice, see, e.g., Hiei et al., 1997.

The Latin names for these and other plants are given below. It should be clear that these and other (non *Agrobacterium*) transformation techniques can be used to transform AAD-12 (v1), for example, into these and other plants, including but not limited to Maize (*Zea mays*), Wheat (*Triticum* spp.), Rice (*Oryza* spp. and *Zizania* spp.), Barley (*Hordeum* spp.), Cotton (*Abroma augusta* and *Gossypium* spp.), Soybean (*Glycine max*), Sugar and table beets (*Beta* spp.), Sugar cane (*Arenga pinnata*), Tomato (*Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato (*Solanum tuberosum*), Sweet potato (*Ipomoea batatas*), Rye (*Secale* spp.), Peppers (*Capiscum annuum*, *sinense*, and *frutescens*), Lettuce (*Lactuca sativa*, *perennis*, and *pulchella*), Cabbage (*Brassica* spp), Celery (*Apium graveolens*), Eggplant (*Solanum melongena*), Peanut (*Arachis hypogea*), Sorghum (all *Sorghum* species), Alfalfa (*Medicago sativua*), Carrot (*Daucus carota*), Beans (*Phaseolus* spp. and other genera), Oats (*Avena sativa* and *strigosa*), Peas (*Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Helianthus annuus*), Squash (*Cucurbita* spp.), Cucumber (*Cucumis sativa*), Tobacco (*Nicotiana* spp.), Ara-

US 8,283,522 B2

83

*bidopsis* (*Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, *Poa*, *Cynodon*, and other genera), Clover (*Tifolium*), Vetch (*Vicia*). Such plants, with AAD-12 (v1) genes, for example, are included in the subject invention.

AAD-12 (v1) has the potential to increase the applicability of key auxinic herbicides for in-season use in many deciduous and evergreen timber cropping systems. Triclopyr, 2,4-D, and/or fluoroxypr resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited to: Alder (*Alnus* spp.), ash (*Fraxinus* spp.), aspen and poplar species (*Populus* spp.), beech (*Fagus* spp.), birch (*Betula* spp.), cherry (*Prunus* spp.), eucalyptus (*Eucalyptus* spp.), hickory (*Carya* spp.), maple (*Acer* spp.), oak (*Quercus* spp.), and pine (*Pinus* spp.). Use of auxin resistance for the selective weed control in ornamental and fruit-bearing species is also within the scope of this invention. Examples could include, but not be limited to, rose (*Rosa* spp.), burning bush (*Euonymus* spp.), petunia (*Petunia* spp.), begonia (*Begonia* spp.), rhododendron (*Rhododendron* spp.), crabapple or apple (*Malus* spp.), pear (*Pyrus* spp.), peach (*Prunus* spp.), and marigolds (*Tagetes* spp.).

#### Example 14

##### Further Evidence of Surprising Results: AAD-12 vs. AAD-2

##### 14.1—AAD-2 (v1) Initial Cloning.

Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to tfdA. This gene is referred to herein as AAD-2 (v1) for consistency. Percent identity was determined by first translating both the AAD-2 and tfdA DNA sequences (SEQ ID NO:12 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

The strain of *Bradyrhizobium japonicum* containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward (SEQ ID NO:16): 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(spel) SEQ ID NO:14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse (SEQ ID NO:17): 5' TIC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 of PCT/US2005/014737 (added a Xho I site)].

Ten of the resulting white colonies were picked into 3 µl Luria Broth+1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C. with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was

84

completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA G 3'] (SEQ ID NO:6) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:7) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation AAD-2 (v1) for internal consistency.

##### 14.2—Completion of AAD-2 (v1) Binary Vector.

The AAD-2 (v1) gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. See PCT/US2005/014737. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:14) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:15) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was then cloned into the pCR2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI AAD-2 (v1) gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: AAD-2 (v1); Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI-NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: AAD-2 (v1); Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR was restriction digested (with Nod, EcoRI, HindIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

##### 14.3—Evaluation of Transformed *Arabidopsis*.

Freshly harvested T<sub>1</sub> seed transformed with a plant optimized AAD-12 (v1) or native AAD-2 (v1) gene were planted and selected for resistance to glufosinate as previously described. Plants were then randomly assigned to various rates of 2,4-D (50-3200 g ae/ha). Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.) mixed in 200 mM Tris buffer (pH 9.0) or 200 mM HEPES buffer (pH7.5).

AAD-12 (v1) and AAD-2 (v1) did provide detectable 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T<sub>1</sub> *Arabidopsis* plants. Surprisingly, AAD-2 (v1) and AAD-2 (v2) transformants were far less resistant to 2,4-D than the AAD-12 (v1) gene, both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with AAD-2 (v1) survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (see PCT/US2005/014737). Conversely, AAD-12 (v1) had a population injury average of about 6% when treated with 3,200 g ae/ha 2,4-D (Table 11). Tolerance improved slightly for plant-optimized AAD-2 (v2) versus the native gene; however, comparison of both AAD-12 and AAD-2 plant optimized genes indicates a significant advantage for AAD-12 (v1) in planta.

US 8,283,522 B2

85

These results are unexpected given that the in vitro comparison of AAD-2 (v1) (see PCT/US2005/014737) and AAD-12 (v2) indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. AAD-2 (v1) is expressed in individual T<sub>1</sub> plants to varying levels; however, little protection from 2,4-D injury is afforded by this expressed protein. No substantial difference was evident in protein expression level (in planta) for the native and plant optimized AAD-2 genes (see PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of AAD-12 (v1) in planta, and resulting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides, unexpected.

## Example 15

## Preplant Burndown Applications

This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-12 invention.

Preplant burndown herbicide applications are intended to kill weeds that have emerged over Winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280-1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2005 or online at [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)). With AAD-12 (v1) transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone+2,4-D or glufosi-

86

nate+2,4-D by utilizing products described in federal herbicide labels (CPR, 2005) and uses described in Agrilience Crop Protection Guide (2005), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-12 (v1) gene if stably transformed. Likewise, the unique attributes of AAD-12 allowing degradation of triclopyr and fluoroxyppr increase utility by allowing substitution or tank mixes of 70-1120 or 35-560 g ae/ha of triclopyr and fluoroxyppr, respectively, to increase spectrum and/or increase the ability to control perennial or viney weed species.

## Example 16

## In-Crop Use of Phenoxy Auxins Herbicides in Soybeans, Cotton, and Other Dicot Crops Transformed Only with AAD-12 (v1)

AAD-12 (v1) can enable the use of phenoxy auxin herbicides (e.g., 2,4-D and MCPA) and pyridyloxy auxins (triclopyr and fluoroxyppr) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560-1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluoroxyppr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, and fluoroxyppr when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to combine herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy.

A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluoroxyppr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetamide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

Further benefits could include tolerance to 2,4-D, triclopyr or fluoroxyppr required before planting following aryloxyacetic acid auxin herbicide application (see previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D, triclopyr or fluoroxyppr. Dicamba (and many other herbicides) can still be used for the subsequent control of AAD-12 (v1)-transformed dicot crop volunteers.

Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other aryloxy auxin herbicide) crop that would be protected by the



US 8,283,522 B2

87

AAD-12 (v1) gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by AAD-12 (v1) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

## Example 17

In-Crop Use of Phenoxy Auxin and Pyridyloxy  
Auxin Herbicides in AAD-12 (v1) Only Transformed  
Corn, Rice, and Other Monocot Species

In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-12 (v1) would allow the use of highly efficacious phenoxy and pyridyloxy auxins in crops where normally selectivity is not certain. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing or unacceptable injury risk. AAD-12 (v1)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluoroxyppy, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, or fluoroxyppy. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-12 (v1) HTC combination strategy, whether one rotates crops species or not.

A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluoroxyppy) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetamide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

The increased tolerance of corn, rice, and other monocots to the phenoxy or pyridyloxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the

88

potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots. Each alternative herbicide enabled for use in HTCs by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

## Example 18

AAD-12 (v1) Stacked With Glyphosate Tolerance  
Trait in Any Crop

The vast majority of cotton, canola, corn, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, petunia, and begonias; [isb.vt.edu/cfdocs/fieldtests1.cfm](http://isb.vt.edu/cfdocs/fieldtests1.cfm), 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking AAD-12 (v1) with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-12 (v1), monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and a GT trait are stacked in any monocot or dicot crop species:

- a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (e.g., *Comelina* spp, *Ipomoea* spp, etc), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluoroxyppy, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.
- b) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. AAD-12 (v1)+GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. Triclopyr and fluoroxyppy at rates mentioned above would be accept-

US 8,283,522 B2

89

able components in the treatment regimen. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D, triclopyr, or fluoroxyppy.

One skilled in the art of weed control will recognize that use of one or more commercial aryloxy auxin herbicides alone or in combination (sequentially or independently) is enabled by AAD-12 (v1) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

#### Example 19

##### AAD-12 (v1) Stacked with Glufosinate Tolerance Trait in Any Crop

Glufosinate tolerance (PAT or bar) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (e.g., rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agrilience, 2005).

By stacking AAD-12 (v1) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

- a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.
- i) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis*, *Apocynum cannabinum*, and *Conyza canadensis*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species. Triclopyr and fluoroxyppy would be acceptable components to consider in the weed control regimen. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g

90

ae/ha. For fluoroxyppy, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

- b) A multiple combination of glufosinate (200-500 g ae/ha)+/-2,4-D (280-1120 g ae/ha)+/-triclopyr or fluoroxyppy (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of one or more commercial aryloxyacetic auxin herbicides alone or in combination (sequentially or independently) is enabled by AAD-12 (v1) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

#### Example 20

##### AAD-12 (v1) Stacked with AHAS Trait in Any Crop

Imidazolinone herbicide tolerance (AHAS, et al.) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapic has been enabled through imidazolinone tolerance traits like AHAS et al. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agrilience, 2005). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2005). By stacking AAD-12 (v1) with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-12 (v1), monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and these auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

- a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.
- i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis*, *Ambrosia trifida*, *Chenopodium album* (among others, Heap, 2005) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. For triclopyr, application rates

US 8,283,522 B2

91

would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluoroxypr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 g ae/ha.

ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea* spp. can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. See rates above for triclopyr or fluoroxypr.

b) A multiple combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha)+/-2,4-D (280-1120 g ae/ha)+/-triclopyr or fluoroxypr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by AAD-12 (v1) transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

#### Example 21

##### AAD-12 (v1) in Rice

###### 21.1—Media Description.

Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/L Phytigel (Sigma). Embryogenic calli were cultured in 100×20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25-26° C., and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang et al. 1996).

Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li et al. 1993), but adapted to contain 500 mg/L glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang et al. 1998) with the inclusion of 30 g/L sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/L hygromycin B for 3-4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/L 6-benzylaminopurine (BAP), 1 mg/L  $\alpha$ -naphthaleneacetic acid (NAA), 5 mg/L abscisic acid (ABA) and 50 mg/L

92

hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/L BAP, 0.5 mg/L NAA, and 50 mg/L hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/L hygromycin B ( $\frac{1}{2}$ MSH50).

###### 21.2—Tissue Culture Development.

Mature desiccated seeds of *Oryza sativa* L. *japonica* cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing  $\frac{1}{2}$  MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on  $\frac{1}{2}$  MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week. prior to storage.

###### 21.3—Microprojectile Bombardment.

All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed one with 100% ethanol, twice with sterile distilled water and resuspended in 50  $\mu$ l water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303 (Hpt-containing vector) to pDAB4101 (AAD-12 (v1)+AHAS), 20  $\mu$ l spermidine (0.1 M) and 50  $\mu$ l calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60  $\mu$ l cold 100% ethanol and 8-9  $\mu$ l was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang et al. (1996).



#### 21.4—Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T<sub>0</sub> Rice

Rice plantlets at the 3-5 leaf stage were sprayed with a lethal dose of 0.16% (v/v) solution of Pursuit (to confirm the presence of the AHAS gene) containing 1% Sunit II (v/v) and 1.25% UAN (v/v) using a track sprayer calibrated to 187 L/ha. Rating for sensitivity or resistance was performed at 36 days after treatment (DAT). Ten of the 33 events sent to the greenhouse were robustly tolerant to the Pursuit; others suffered varying levels of herbicide injury. Plants were sampled (according to section 21.7 below) and molecular characterization was performed as previously described in Example 8 that identified seven of these 10 events as containing both the AAD-12 (v1) PTU and the entire AHAS coding region.

#### 21.5—Heritability of AAD-12 (v1) in T<sub>1</sub> Rice

A 100-plant progeny test was conducted on five T<sub>1</sub> lines of AAD-12 (v1) lines that contained both the AAD-12 (v1) PTU and AHAS coding region. The seeds were planted with respect to the procedure above and sprayed with 140 g ae/ha imazethapyr using a track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Two out of the five lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 coseregated with the AHAS selectable marker as determined by 2,4-D tolerance testing below.

#### 21.6—Verification of High 2,4-D Tolerance in T<sub>1</sub> Rice.

The following T<sub>1</sub> AAD-12 (v1) single segregating locus lines were planted into 3-inch pots containing Metro Mix media: pDAB4101(20)003 and pDAB4101(27)002. At 2-3 leaf stage were sprayed with 140 g ae/ha imazethapyr. Nulls were eliminated and individuals were sprayed at V3-V4 stage in the track sprayer set to 187 L/ha at 1120, 2240 or 4480 g ae/ha 2,4-D DMA (2×, 4×, and 8× typical commercial use rates, respectively). Plants were graded at 7 and 14 DAT and compared to untransformed commercial rice cultivar, 'Lamont,' as negative control plants.

Injury data (Table 27) shows that the AAD-12 (v1)-transformed lines are more tolerant to high rates of 2,4-D DMA than the untransformed controls. The line pDAB4101(20)003 was more tolerant to high levels of 2,4-D than the line pDAB4101(27)002. The data also demonstrates that tolerance of 2,4-D is stable for at least two generations.

TABLE 27

T <sub>1</sub> AAD-12 (v1) and untransformed control response to varying levels of 2,4-D DMA.			
Herbicide	Lemont Untransformed Control	pDAB4101(20)003	pDAB4101(27)002
		Average % Injury 14 DAT	
1120 g ae/ha 2,4-D DMA	20	10	10
2240 g ae/ha 2,4-D DMA	35	15	30
4480 g ae/ha 2,4-D DMA	50	23	40

#### 21.7—Tissue Harvesting, DNA Isolation and Quantification.

Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then

followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μl.

#### 21.8—AAD-12 (v1) Expression.

Sample preparation and analysis conditions were as described previously. All 33 T<sub>0</sub> transgenic rice lines and 1 non-transgenic control were analyzed for AAD-12 expression using ELISA blot. AAD-12 was detected in the clones of 20 lines, but not in line Taipai 309 control plant. Twelve of the 20 lines that had some of the clones tolerant to imazethapyr were expressing AAD-12 protein, were AAD-12 PCR PTU positive, and AHAS coding region positive. Expression levels ranged from 2.3 to 1092.4 ppm of total soluble protein.

#### 21.9—Field Tolerance of pDAB4101 Rice Plants to 2,4-D and Triclopyr Herbicides.

A field level tolerance trial was conducted with AAD-12 (v1) event pDAB4101[20] and one wild-type rice (Clearfield 131) at Wayside, Miss. (a non-transgenic imidazolinone-resistant variety). The experimental design was a randomized complete block design with a single replication. Herbicide treatments were 2× rates of 2,4-D (dimethylamine salt) at 2240 g ae/ha and triclopyr at 560 g ae/ha plus an untreated control. Within each herbicide treatment, two rows of T<sub>1</sub> generation pDAB4101[20] and two rows of Clearfield rice were planted using a small plot drill with 8-inch row spacing. The pDAB4101 [20] rice contained the AHAS gene as a selectable marker for the AAD-12(v1) gene. Imazethapyr was applied at the one leaf stage as selection agent to remove any AAD-12 (v1) null plants from the plots. Herbicide treatments were applied when the rice reached the 2 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual ratings of injury were taken at 7, 14 and 21 days after application.

AAD-12 (v1) event response to 2,4-D and triclopyr are shown in Table 28. The non-transformed rice line (Clearfield) was severely injured (30% at 7DAT and 35% at 15DAT) by 2,4-D at 2240 g ae/ha which is considered the 4× commercial use rate. The AAD-12 (v1) event demonstrated excellent tolerance to 2,4-D with no injury observed at 7 or 15DAT. The non-transformed rice was significantly injured (15% at 7DAT and 25% at 15DAT) by the 2× rate of triclopyr (560 g ae/ha). The AAD-12 (v1) event demonstrated excellent tolerance to the 2× rates of triclopyr with no injury observed at either 7 or 15DAT.

These results indicate that the AAD-12 (v1) transformed rice displayed a high level of resistance to 2,4-D and triclopyr at rates that caused severe visual injury to the Clearfield rice. It also demonstrates the ability to stack multiple herbicide tolerance genes with AAD-12 1 multiple species to provide resistance to a wider spectrum of effective chemistries

US 8,283,522 B2

95

96

TABLE 28

AAD-12 T <sub>1</sub> generation rice plants response to 2,4-D and triclopyr under field conditions.					
Herbicide Treatment		% Visual Injury			
		7DAT		15DAT	
Active Ingredient	Rate	AAD-12 event pDAB4101[20]	Wild-type Clearfield	AAD-12 event pDAB4101[20]	Wild-type Clearfield
2,4-D	2240 GM AE/HA	0	15	0	35
Triclopyr	840 GM AE/HA	0	30	0	25
Untreated		0	0	0	0

## Example 22

## AAD-12 (v1) in Canola

## 22.1—Canola Transformation.

The AAD-12 (v1) gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. *Nexera*\*710 with *Agrobacterium*-mediated transformation and plasmid pDAB3759. The construct contained AAD-12 (v1) gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter and the EPSPS glyphosate resistance trait driven by AtUbi10 promoter (see section 2.4).

Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C., and a photoperiod of 16 hrs light/8 hrs dark.

Hypocotyl segments (3-5 mm) were excised from 5-7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/L kinetin and 1 mg/L 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB3759. The *Agrobacterium* was grown overnight at 28° C. in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed on K1D1TC (callus induction medium containing 250 mg/L Carbenicillin and 300 mg/L Timentin) for one week or two weeks of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/L Herbiace). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbiace allowed the growth of the transformed cells.

Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 1 mg/L Herbiace, Carbenicillin and Timentin) shoot regeneration medium. After 2-3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 3 mg/L Herbiace, Carbenicillin and Timentin) for another 2-3 weeks.

Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH5 or MES10 (MS, 0.5 gm/L MES, 5 or 10 mg/L Herbiace, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured

for root induction on MS1.1 (MS with 0.1 mg/L Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

## 22.2—Molecular Analysis: Canola Materials and Methods

22.2.1—Tissue harvesting DNA isolation and quantification. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μl.

22.2.2—Polymerase chain reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR AAD-12 (v1) were (SEQ ID NO:10) (forward) and (SEQ ID NO:11) (reverse). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 2 minutes followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with AAD-12 (v1) events tested positive. Three negative control samples tested negative.

## 22.2.3—ELISA.

Using established ELISA described in previous section, AAD-12 protein was detected in 5 different canola transformation plant events. Expression levels ranged from 14 to over 700 ppm of total soluble protein (TSP). Three different untransformed plant samples were tested in parallel with no signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. These samples were also confirmed positive by Western analysis. A summary of the results is presented in Table 29.

TABLE 29

Expression of AAD-12 (v1) in Canola plants				
Sample #	[TSP] (μg/ml)	[AAD-12] (ng/ml)	Expression (ppm TSP) (ELISA)	Western
31	5614.96	1692.12	301.36	++++
33	4988.26	2121.52	425.30	++++
38	5372.25	3879.09	722.06	++++
39	2812.77	41.36	14.71	+

## US 8,283,522 B2

97

TABLE 29-continued

Expression of AAD-12 (v1) in Canola plants				
Sample #	[TSP] (µg/ml)	[AAD-12] (ng/ml)	Expression (ppm TSP) (ELISA)	Western
40	3691.48	468.74	126.98	+++
Control 1	2736.24	0.00	0.00	-
Control 2	2176.06	0.00	0.00	-
Control 3	3403.26	0.00	0.00	-

22.4—Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T<sub>0</sub> Canola.

Forty-five T<sub>0</sub> events from the transformed with the construct pDAB3759, were sent to the greenhouse over a period of time and were allowed to acclimate in the greenhouse. The plants were grown until 2-4 new, normal looking leaves had emerged (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were then treated

98

22.6—Verification of High 2,4-D Tolerance in T<sub>1</sub> Canola

For T<sub>1</sub> AAD-12 (v1), 5-6 mg of seed were stratified, sown, and a fine layer of Sunshine Mix #5 media was added as a top layer of soil. Emerging plants were selected with 560 g ae/ha 2,4-D at 7 and 13 days after planting.

Surviving plants were transplanted into 3-inch pots containing Metro Mix media. Surviving plants from T<sub>1</sub> progenies, that were selected with 560 g ae/ha 2,4-D, were also transplanted into 3-inch pots filled with Metro Mix soil. At 2-4 leaf stage plants were sprayed with either 280, 560, 1120, or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to untransformed control plants. A sampling of T<sub>1</sub> event injury data 14DAT may be seen in Table 30. Data suggests that multiple events are robustly resistant to 2240 g ae/ha 2,4-D, while other events demonstrated less robust tolerance up to 1120 g ae/ha 2,4-D. Surviving plants were transplanted to 5¼" pots containing Metro Mix media and placed in the same growth conditions as before and self-pollinated to produce only homozygous seed.

TABLE 30

T <sub>1</sub> AAD-12 (v1) and untransformed control response to varying rates postemergence 2,4-D DMA applications.						
Herbicide	Un-transformed Control	pDAB3759(33)013.001	pDAB3759(18)009.001	pDAB3759(18)022.001	pDAB3759(18)030.001	pDAB3759(18)023.001
Average % Injury 14DAT						
280 g ae/ha 2,4-D DMA	85	0	0	0	0	0
560 g ae/ha 2,4-D DMA	85	0	0	0	0	0
1120 g ae/ha 2,4-D DMA	90	0	0	13	5	3
2240 g ae/ha 2,4-D DMA	95	1	5	83	31	6

with a lethal dose of the commercial formulations of 2,4-D Amine 4 at a rate of 560 g ae/ha. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. A lethal dose is defined as the rate that causes >95% injury to the untransformed controls.

Twenty-four of the events were tolerant to the 2,4-D DMA herbicide application. Some events did incur minor injury but recovered by 14 DAT. Events were progressed to the T<sub>1</sub> (and T<sub>2</sub> generation) by selfpollination under controlled, bagged, conditions.

## 22.5—AAD-12 (v1) Heritability in Canola.

A 100 plant progeny test was also conducted on 11 T<sub>1</sub> lines of AAD-12 (v1). The seeds were sown and transplanted to 3-inch pots filled with Metro Mix media. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Seven out of the 11 lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi-square analysis. AAD-12 is heritable as a robust aryloxy-alkanoate auxin resistance gene in multiple species and can be stacked with one or more additional herbicide resistance genes.

45

## 22.7—Field Tolerance of pDAB3759 Canola Plants to 2,4-D, Dichloprop, Triclopyr and Fluoroxypyr Herbicides.

Field level tolerance trial was conducted on two AAD-12 (v1) events 3759(20)018.001 and 3759(18)030.001 and a wild-type canola (Nex710) in Fowler, Ind. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluoroxypyr at 280 g ae/ha and an untreated control. Within each herbicide treatment, single 20 ft row/event for event 3759(18)030.001, 3759(18)018.001 and wild-type line (Nex710) were planted with a 4 row drill on 8 inch row spacing. Herbicide treatments were applied when canola reached the 4-6 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after application.

Canola response to 2,4-D, triclopyr, and fluoroxypyr are shown in Table 31. The wild-type canola (Nex710) was severely injured (72% at 14DAT) by 2,4-D at 2240 g ae/ha which is considered the 4× rate. The AAD-12 (v1) events all demonstrated excellent tolerance to 2,4-D at 14DAT with an

US 8,283,522 B2

99

average injury of 2, 3 and 2% observed at the 1, 2 and 4× rates, respectively. The wild-type canola was severely injured (25% at 14DAT) by the 2× rate of triclopyr (840 g ae/ha). AAD-12 (v1) events demonstrated tolerance at 2× rates of triclopyr with an average of 6% injury at 14DAT across the two events. Fluoroxypyr at 280 g ae/ha caused severe injury (37%) to the non-transformed line at 14DAA. AAD-12 (v1) events demonstrated increased tolerance with an average of 8% injury at 5DAT.

These results indicate that AAD-12 (v1) transformed events displayed a high level of resistance to 2,4-D, triclopyr and fluoroxypyr at rates that were lethal or caused severe epinastic malformations to non-transformed canola. AAD-12 has been shown to have relative efficacy of 2,4-D>triclopyr>fluoroxypyr.

TABLE 31

AAD-12 (pDAB3759) canola plants response to 2,4-D, triclopyr, and fluoroxypr under field conditions.				
Herbicide Treatment		% Visual Injury at 14 DAT		
Active Ingredient	Rate	AAD-12 event 3759(20)018.001	AAD-12 event 3759(18)030.001	Wild Type (Nex710)
2,4-D	280 GM AE/HA	0 a	0 b	0 e
2,4-D	560 GM AE/HA	0 a	0 b	15 d
2,4-D	1120 GM AE/HA	2 a	2 ab	33 bc
2,4-D	2240 GM AE/HA	3 a	3 ab	48 a
Triclopyr	840 GM AE/HA	6 a	6 ab	25 cd
Fluroxypyr	280 GM AE/HA	7 a	8 a	37 ab

Means with a column with different letters are significantly different as defined by LSD (p = 0.05).

100

of weed control will recognize that use of any of various commercial herbicides described in Examples 18-20, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by AAD-12 (v1) transformation and stacking with the corresponding HT trait or IR trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

## Example 23

## AAD-12 (v1) Stacked With Insect Resistance (IR) or Other Input Traits in Any Crop

Insect resistance in crops supplied by a transgenic trait is prevalent in corn and cotton production in North America and across the globe. Commercial products having combined IR and HT traits have been developed by multiple seed companies. These include Bt IR traits (e.g. Bt toxins listed at the website [lifesci.sussex.ac.uk](http://lifesci.sussex.ac.uk), 2006) and any or all of the HTC traits mentioned above. The value this offering brings is the ability to control multiple pest problems through genetic means in a single offering. The convenience of this offering will be restricted if weed control and insect control are accomplished independent of each other. AAD-12 (v1) alone or stacked with one or more additional HTC traits can be stacked with one or more additional input traits (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) (isb.vt.edu/cfdocs/fieldtests1.cfm, 2005) either through conventional breeding or jointly as a novel transformation event. Benefits include the convenience and flexibility described in Examples 15-20 above, together with the ability to manage insect pests and/or other agronomic stresses in addition to the improved weed control offered by AAD-12 and associated herbicide tolerance. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic issues.

Combined traits of IR and HT have application in most agronomic and horticultural/oramental crops and forestry. The combination of AAD-12 and its commensurate herbicide tolerance and insect resistance afforded by any of the number of Bt or non-Bt IR genes can be applied to the crop species listed (but not limited to) in Example 13. One skilled in the art

## Example 24

## AAD-12 (v1) as an in vitro Dicot Selectable Marker

Genetic engineering of plant cell, tissue, organ, and plant or organelle such as plastid starts with the process of inserting genes of interest into plant cells using a suitable delivery method. However, when a gene is delivered to plant cells, only an extremely small percentage of cells integrate the heterogeneous gene into their genome. In order to select those few cells that have incorporated the gene of interest, researchers link a selectable or screenable “marker gene” to the gene of interest (GOI) in the vector. Cells that contain these markers are identified from the whole population of cells/tissue to which the DNA plasmid vector was delivered. By selecting those cells that express the marker gene, researchers are able to identify those few cells that may have incorporated the GOI into their genome.

There are a variety of selectable markers available to enable this selection process to obtain transgenic cells, callus, embryos, shoots and plantlets. The preferred selectable markers by the Ag-industry are herbicide markers that allow the ease of spraying compounds in the field to select the right transgenic progenies during the process of event sorting in the field situation. AAD-12 (v1) has been shown to efficiently serve as a selectable marker for whole plants transformed with the gene in the greenhouse and growth chamber (Example 7) with 2,4-D as the selection agent. Field selection is possible as well using 2,4-D in combination with the AAD-12 (v1) gene (Example 11, 22), but use in vitro for cell-level selection is complicated by the fact 2,4-D is used almost ubiquitously as a plant growth regulator in the plant tissue culture systems. Degradation of this important hormone by AAD-12 (v1) can impact the ability to use this gene as an in vitro selectable marker. Success of developing 2,4-D as a



US 8,283,522 B2

101

marker gene depends on identifying the right alternate plant growth regulator that can mimic the effect of 2,4-D in the respective culture system and at the same time possess the ability to be stable and not be degraded by the AAD-12 enzyme when expressed in the transgenic cells. R-dichlorprop is a close analog to 2,4-D that is not a substrate for AAD-12 (v1) and is used as a non-metabolizable auxin substitute in tobacco cell cultures allowing 2,4-D to be used at high rates as a selection agent. This fact was used in exemplifying AAD-12 (v1) could be used as a selectable marker in vitro.

#### 24.1—Cell Culture—Alternative Auxins.

AAD-12 (v1) degrades 2,4-D, but not R-2,4-dichlorophenoxypropionic acid (R-dichlorprop), which has at the same time the structural requirement of an auxinic growth regulator. Other non-metabolizable plant auxin mimics that may be used in cell culture include NAA (naphthalene acetic acid), IAA (indole acetic acid), dicamba, picloram, and R-mecoprop. It was investigated if it was possible to substitute R-dichlorprop and successfully maintain two different tobacco cell cultures PHL (Petite Havanna) and BY2 suspensions. Conversely, for cotton explants R-dichlorprop, dicamba, and picloram were tested as alternative auxins and the embryogenic callus induction response in comparison to the standard growth regulator, 2,4-D was evaluated. Petite Havana tobacco (PHL) and Coker cotton cotyledons were used in their experiments.

##### 24.1.1—Tobacco Cell Suspension—2,4-D as Selection Agent.

A dose response study was conducted with both the R-dichlorprop habituated PHL cells and the R-dichlorprop habituated BY2 cells where R-dichlorprop was substituted directly for 2,4-D in culture media. Though the focus was on PHL, a dose response was also done with BY2 in case of possible future studies, as well as to help predict the dose response for PHL. For the dichlorprop habituated PHL dose response, the levels of 2,4-D used (on LSBY2C medium with R-dichlorprop) were 0 (the control), 1, 2, 3, 5, 8, 10, 12, 15, 18, 20, 40, 60, 80, 100, 110, 120 mg/L 2,4-D. There were four replications per concentration. For the R-dichlorprop habituated BY2 dose response, the levels of 2,4-D used (on LSBY2C medium) were 0 (the control), 1, 2, 3, 5, 8, 10, 20, 30, 40, mg/L 2,4-D.

The dose response was carried out showed that all the concentration of 2,4-D tested were lethal above 10 mg/L concentrations. However, there was growth in all the concentrations up to 10-mg/L 2,4-D where a slight growth of PHL suspension was observed. The growth of the suspension colonies from 1-8 mg/L 2,4-D concentrations was comparable to the growth in control treatments. The observation made in BY2 suspension cells were similar except the concentration at 10 mg/L was found to be lethal and the sub lethal concentration was 8 mg/L concentration.

##### 24.1.2—Tobacco Cell Transformation with AAD-12 (v1) and 2,4-D Selection.

For tobacco transformation experiment, there were 11 treatments altogether: a control set plated on LS-BY2C+ dichlorprop medium, and 10 sets of LSBY2C+ dichlorprop+ 2,4-D at varying concentration levels (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L). There were four replications per treatment. The plasmid DNA vector used was pDAB724, and the vector used for transformation was EHA101S strain of *Agrobacterium tumefaciens*. Four ml of PHL suspension at  $0.6 \text{ OD}^{660}$  were mixed with 100  $\mu\text{L}$  of *Agrobacterium* (either EHA101 or LBA4404 strains) suspension at  $1.0 \text{ OD}^{660}$  in a sterile Petri plate and were mixed thoroughly and co-cultivated together in a non-shake condition at a dark growth chamber for 3 days at  $25^\circ \text{C}$ . After the co-cultivation period 1.5 ml of the Agro-

102

tobacco suspension mixture was plated to the 11 set of plates above. The experiment was repeated with 13 treatments: a control of LS-BY2C+dichlorprop media (no 2,4-D), and LS-BY2C+dichlorprop+2,4-D (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L); LSBY2C+1 mg/L 2,4-D+B10 (Bialophos); LSBY2C+10 2,4-D+B 10+R-dichlorprop. Again, there were four replicates per treatment, as well as a positive and negative control. All media contain 500 mg/L Carbenicillin (C) to control to contain *Agrobacterium* growth in the selection media.

The plasmid used in these experiments is pDAB724 and it has PAT selectable marker as well. So, control transformation experiments were initiated using R-dichlorprop habituated PHL in the presence of 10 mg/L bialophos following the standard protocol described above. The treatments were done side by side with 4 replicates to see if the bialophos selection in these suspension is normal.

There was little growth observed in all selection concentrations of 2,4D tested above 10 mg/L; however several fast growing colonies were found in 2, 5, an 8 mg/L 2,4-D concentration and representative sample was transferred to fresh selection at 10 mg/L selection to bulk the callus. Also, several putative colonies were selected in from 12, 15, 18 and 20 mg/L 2,4-D, but when compared to 10 mg/L there were only few colonies in these selection plate. Control treatment conducted with bialophos selection showed normal colony development. It appears that 10 mg/L 2,4-D is the sub-lethal and above this concentration 2,4-D appears to be lethal to the non-transformed cells. All the identified colonies were transferred to fresh medium with 10 mg/L selection and were probed for the presence of transgene by PCR as described in Example 10. The colonies selected and bulked had the transgenes as determined by PCR and expression of the genes as established by the Western analyses (as described in example 10). Several colonies were identified as actively growing and transferred to fresh selection medium with 10 mg/L 2,4-D to bulk the callus.

The bulked calluses were then transferred to higher level of 2,4-D to test the tolerance level in vitro. The levels of 2,4-D used were 20, 40, 60, 80, 100, and 120 mg/L 2,4-D. However the callus did not grow beyond 20 mg/L 2,4-D concentrations indicating a threshold concentration higher than 20 mg/L may exist.

##### 24.2.1—Cotton Explants—Auxin Alternatives

A dose response study was initiated to test multiple auxin alternatives as a substitute for the use of 2,4-D as a growth regulator in cotton. The alternative auxin tested were 2,4-dichlorprop, dicamba, and picloram. These compounds were tested at 0.2, 2.0, and 20.0  $\mu\text{M}$  concentrations respectively. 2,4-D was used as the control treatment at 0.02  $\mu\text{M}$  concentration. The medium used is the base medium for cotton callus induction (Example 12). Beyond the initial phase of culture, auxin is removed from the medium to prod the tissue toward the regeneration process.

R-dichlorprop was not effective in callus induction of cotyledonary segments and appears toxic to cotton cells at the lowest concentration tested (0.02  $\mu\text{M}$ ). Dicamba effectively induces callus growth at all concentrations tested (0.02-20  $\mu\text{M}$ ) and has no apparent toxic effects in this concentration range. Callus induction with picloram increased up to a maximum when explants were treated with 0.2  $\mu\text{M}$  to 20  $\mu\text{M}$ . Quality of the callus was consistent with the standard 2,4-D treatment at the 2  $\mu\text{M}$  picloram concentration. At the highest concentration (20  $\mu\text{M}$ ) 2,4-D was also inhibitory to cotton callus generation and growth.

Cotton has shown initial ability to respond effectively to alternative auxins (to 2,4-D) in culture. At high enough con-

US 8,283,522 B2

103

centrations, 2,4-D is toxic to cotton cotyledonary explants. R-dichlorprop is surprisingly significantly more toxic to cotton than 2,4-D or other auxins. 2,4-D may be used as a selection agent and in combination with AAD-12 (v1) as the selectable marker gene. Other non-metabolizable auxin surrogates (e.g., dicamba, picloram, R-mecoprop, NAA, or IAA) would allow the use of AAD-12 as a selectable marker in dicots with 2,4-D as the selection agent.

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US 8,283,522 B2

105

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107

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108

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## SEQUENCE LISTING

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&lt;213&gt; ORGANISM: Delftia acidovorans

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gtgcacctgg ccacgctgga cgacgcgggc ttgcgcgcc tgcacgccgc ctggtgcag 120

catgcgctgc tgatottccc cggccagcac ctcagcaacg accagcagat cacttttgcc 180

US 8,283,522 B2

109

110

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aaacgcttcg gcgcgatcga gcgcacggc ggccggcgaca tcgtggccat ctccaatgtc 240
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Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro Gly
35     40     45
Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe Gly
50     55     60
Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val Ala Ile Ser Asn Val
65     70     75     80
Lys Ala Asp Gly Thr Val Arg Gln His Ser Pro Ala Glu Trp Asp Asp
85     90     95
Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser Thr
100    105    110
Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val Val
115    120    125
Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala Tyr
130    135    140
Asp Ala Leu Asp Glu Ala Thr Arg Ala Leu Val His Gln Arg Ser Ala
145    150    155    160
Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln Gln
165    170    175
Ala Gly Ser Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr Pro
180    185    190
Leu Arg Pro Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser Leu
195    200    205
Leu Ile Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala Glu
210    215    220
Ser Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala Pro
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Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp Asp
245    250    255

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US 8,283,522 B2

111

112

-continued

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<213> ORGANISM: Delftia acidovorans

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Gly Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe  
50 55 60

Gly Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val Ala Ile Ser Asn  
65 70 75 80

Val Lys Ala Asp Gly Thr Val Arg Gln His Ser Pro Ala Glu Trp Asp  
85 90 95

Asp Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser  
100 105 110

Thr Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val  
115 120 125

Val Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala

US 8,283,522 B2

113

114

-continued

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Ala Arg His Ser	Leu Val Tyr Ser Gln Ser	Lys Leu Gly His Val Gln
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180	185	190
Pro Leu Arg Pro	Leu Val Lys Val His Pro	Glu Thr Gly Arg Pro Ser
195	200	205
Leu Leu Ile Gly	Arg His Ala His Ala Ile	Pro Gly Met Asp Ala Ala
210	215	220
Glu Ser Glu Arg	Phe Leu Glu Gly Leu Val	Asp Trp Ala Cys Gln Ala
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Pro Arg Val His	Ala His Gln Trp Ala Ala	Gly Asp Val Val Val Trp
245	250	255
Asp Asn Arg Cys	Leu Leu His Arg Ala Glu	Pro Trp Asp Phe Lys Leu
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290		

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US 8,283,522 B2

115

116

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US 8,283,522 B2

117

118

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We claim:

1. A plant cell comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.
2. A plant comprising a plurality of cells of claim 1.
3. A method of controlling weeds in a crop field, said method comprising applying an aryloxyalkanoate herbicide to said crop field, said crop field comprising a plurality of plants, each said plant comprising a plurality of plant cells of claim 1, wherein expression of said polynucleotide renders said plant resistant or tolerant to said aryloxyalkanoate herbicide.
4. The method of claim 3 wherein said aryloxyalkanoate herbicide is 2,4-D.
5. The method of claim 3 wherein said plants are dicots.
6. The method of claim 5 wherein said dicots are soybean plants.
7. The method of claim 3 wherein said method further comprises applying said aryloxyalkanoate herbicide to said crop field prior to planting seeds in said field.
8. The method of claim 3 wherein said method further comprises applying said aryloxyalkanoate herbicide to said crop field after seeds are planted in said field but prior to emergence of said plants grown from said seeds.
9. The method of claim 3 wherein said method further comprises applying glyphosate to said crop field, wherein

US 8,283,522 B2

119

said plants further comprise a second polynucleotide wherein expression of said second polynucleotide renders said plants resistant or tolerant to glyphosate.

10. The method of claim 3 wherein said method further comprises applying glufosinate to said crop field, wherein said plants further comprise a second polynucleotide wherein expression of said second polynucleotide renders said plants resistant or tolerant to glufosinate.

11. The method of claim 3 wherein said method further comprises applying glyphosate and glufosinate to said crop field, wherein said plants further comprise a second polynucleotide wherein expression of said second polynucleotide

120

renders said plants resistant or tolerant to glyphosate, and wherein said plants further comprise a third polynucleotide wherein expression of said third polynucleotide renders said plants resistant or tolerant to glufosinate.

12. The plant cell of claim 1 wherein said protein has at least 99% amino acid sequence identity with SEQ ID NO:2 or SEQ ID NO:4.

13. A seed comprising a plant cell of claim 1.

14. The plant of claim 2 wherein said plant further comprises an insect-resistance gene.

\* \* \* \* \*

UNITED STATES PATENT AND TRADEMARK OFFICE

**CERTIFICATE OF CORRECTION**

PATENT NO. : 8,283,522 B2  
APPLICATION NO. : 12/091896  
DATED : October 9, 2012  
INVENTOR(S) : Terry R. Wright et al.

Page 1 of 1

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In the Claims:

Claim 3

Column 117, line 62, delete "arlyoxyalkanoate" and insert -- aryloxyalkanoate --.

Column 117, line 66, delete "arlyoxyalkanoate" and insert -- aryloxyalkanoate --.

Claim 4

Column 118, line 54, delete "arlyoxyalkanoate" and insert -- aryloxyalkanoate --.

Claim 7

Column 118, line 60, delete "arlyoxyalkanoate" and insert -- aryloxyalkanoate --.

Claim 8

Column 118, line 63, delete "arlyoxyalkanoate" and insert -- aryloxyalkanoate --.

Signed and Sealed this  
Twenty-third Day of July, 2013



Teresa Stanek Rea  
Acting Director of the United States Patent and Trademark Office

# **Exhibit E**

(12) **United States Patent**  
**Bard et al.**

(10) **Patent No.:** **US 8,680,363 B2**  
(45) **Date of Patent:** **Mar. 25, 2014**

(54) **INSECT RESISTANT AND HERBICIDE  
TOLERANT SOYBEAN EVENT 9582.814.19.1**

(75) Inventors: **Nathan Bard**, Edgerton, WI (US);  
**Gregory A. Bradfisch**, Carmel, IN (US);  
**Yunxing C. Cui**, Carmel, IN (US);  
**James E. Dripps**, Carmel, IN (US);  
**Thomas Hoffman**, Zionsville, IN (US);  
**Dayakar Paredy**, Carmel, IN (US);  
**Dawn M. Parkhurst**, Avon, IN (US);  
**Sandra G. Toledo**, West Lafayette, IN  
(US); **Barry Wiggins**, Westfield, IN  
(US); **Ning Zhou**, Zionsville, IN (US)

(73) Assignee: **Dow AgroSciences, LLC.**, Indianapolis,  
IN (US)

(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 0 days.

(21) Appl. No.: **13/559,177**

(22) Filed: **Jul. 26, 2012**

(65) **Prior Publication Data**

US 2013/0061346 A1 Mar. 7, 2013

**Related U.S. Application Data**

(60) Provisional application No. 61/511,664, filed on Jul.  
26, 2011, provisional application No. 61/521,798,  
filed on Aug. 10, 2011.

(51) **Int. Cl.**  
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None

See application file for complete search history.

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(57) **ABSTRACT**

Soybean event 9582.814.19.1, wherein the event comprises  
genes encoding Cry1F, Cry1Ac (synpro), and PAT, affording  
insect resistance and herbicide tolerance to soybean crops  
containing the event, and enabling methods for crop protec-  
tion and protection of stored products.

**9 Claims, 2 Drawing Sheets**

US 8,680,363 B2

1

**INSECT RESISTANT AND HERBICIDE  
TOLERANT SOYBEAN EVENT 9582.814.19.1****CROSS-REFERENCE TO RELATED  
APPLICATION**

This application claims the benefits of U.S. Provisional Application No. 61/511,664, filed Jul. 26, 2011, and U.S. Provisional Application No. 61/521,798, filed Aug. 10, 2011, both of which are herein incorporated by reference in their entireties.

**BACKGROUND OF INVENTION**

The genes encoding Cry1F and Cry1Ac synpro (Cry1Ac) are capable of imparting insect resistance, e.g., resistance to lepidopteran insects, to transgenic plants; and the gene encoding PAT (phosphinothricin acetyltransferase) is capable of imparting tolerance to the herbicide phosphinothricin (glufosinate) to transgenic plants. PAT has been successfully expressed in soybean for use both as a selectable marker in producing insect resistant transgenic crops, and to impart commercial levels of tolerance to the herbicide glufosinate in transgenic crops.

The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site (Weising et al., *Ann. Rev. Genet.* 22:421-477, 1988). At the same time the presence of the transgene at different locations in the genome will influence the overall phenotype of the plant in different ways. For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of an introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant tissues, that may not correspond to the patterns expected from transcriptional regulatory elements present in the introduced gene construct. For this reason, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual outcrossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure reliable gene expression in a number of varieties that are well adapted to local growing conditions.

It is desirable to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene or group of transgenes of interest. In addition, a method for detecting a particular event would be helpful for complying with regulations requiring the pre-market approval and labeling of foods derived from recombinant crop plants, for example, or for use in environmental monitoring, monitoring traits in crops in the field, or monitoring products derived from a crop harvest, as well as for use in ensuring compliance of parties subject to regulatory or contractual terms.

It is possible to detect the presence of a transgenic event by any nucleic acid detection method known in the art including, but not limited to, the polymerase chain reaction (PCR) or

2

DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc., because for many DNA constructs, the coding region is interchangeable. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known. For example, an event-specific PCR assay is described in United States Patent Application 2006/0070139 for maize event DAS-59122-7. It would be desirable to have a simple and discriminative method for the identification of soybean event 9582.814.19.1.

**BRIEF SUMMARY OF THE INVENTION**

The present invention relates to a new insect resistant and herbicide tolerant transgenic soybean transformation event, designated soybean event 9582.814.19.1, comprising cry1F, cry1Ac and pat, as described herein, inserted into a specific site within the genome of a soybean cell. Representative soybean seed has been deposited with American Type Culture Collection (ATCC) with the Accession No. identified in paragraph [0021]. The DNA of soybean plants containing this event includes the junction/flanking sequences described herein that characterize the location of the inserted DNA within the soybean genome. SEQ ID NO:1 and SEQ ID NO:2 are diagnostic for soybean event 9582.814.19.1. More particularly, sequences surrounding the junctions at bp 1400/1401, and bp 1536/1537 of SEQ ID NO:1, and bp 152/153 of SEQ ID NO:2 are diagnostic for soybean event 9582.814.19.1. Paragraph [00012] below describes examples of sequences comprising these junctions that are characteristic of DNA of soybeans containing soybean event 9582.814.19.1.

In one embodiment, the invention provides a soybean plant, or part thereof, that is resistant to *Pseudoplusia includens* (soybean looper) and that has a genome comprising one or more sequences selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof. In another embodiment, the invention provides seed of such plants.

In another embodiment, the invention provides a method of controlling insects that comprises exposing insects to insect resistant soybean plants, wherein the soybean plants have a genome that contains one or more sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof; which are characteristic of the presence of soybean event 9582.814.19.1, to thereby control the insects. Presence of the cry1F v3 (cry1F) and cry1Ac synpro (cry1Ac) genes in soybean event 9582.814.19.1 imparts resistance to, for example, *Pseudoplusia includens* (soybean looper), *Anticarsia gemmatilis* (velvetbean caterpillar), *Epinotia aporema*, *Omoides indicatus*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Spodoptera cosmoides*, *Spodoptera eridania*, *Heliothis virescens*, *Heliooverpa zea*, *Spilosoma virginica* and *Elasmopalpus lignosellus*.

In another embodiment, the invention provides a method of controlling weeds in a soybean crop that comprises applying glufosinate herbicide to the soybean crop, said soybean crop



US 8,680,363 B2

3

comprising soybean plants that have a genome containing one or more sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof, which are diagnostic for the presence of soybean event 9582.814.19.1. Presence of the pat v6 (pat) gene in soybean event 9582.814.19.1 imparts tolerance to glufosinate herbicide.

In another embodiment, the invention provides a method of detecting soybean event 9582.814.19.1 in a sample comprising soybean DNA, said method comprising:

(a) contacting said sample with

a first primer at least 10 bp in length that selectively binds to a flanking sequence within bp 1-1400 of SEQ ID NO:1 or the complement thereof, and a second primer at least 10 bp in length that selectively binds to an insert sequence within bp 1401-1836 of SEQ ID NO:1 or the complement thereof; and assaying for an amplicon generated between said primers; or (b) contacting said sample with a first primer at least 10 bp in length that selectively binds to an insert sequence within bp 1-152 of SEQ ID NO:2 or the complement thereof, and a second primer at least 10 bp in length that selectively binds to flanking sequence within bp 153-1550 of SEQ ID NO:2 or the complement thereof; and

(c) assaying for an amplicon generated between said primers.

In another embodiment, the invention provides a method of detecting soybean event 9582.814.19.1 comprising:

(a) contacting said sample with a first primer that selectively binds to a flanking sequence selected from the group consisting of bp 1-1400 of SEQ ID NO:1 and bp 153-1550 of SEQ ID NO:2, and complements thereof; and a second primer that selectively binds to SEQ ID NO:3, or the complement thereof;

(b) subjecting said sample to polymerase chain reaction; and

(c) assaying for an amplicon generated between said primers.

In another embodiment the invention provides a method of breeding a soybean plant comprising: crossing a first plant with a second soybean plant to produce a third soybean plant, said first plant comprising DNA comprising one or more sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof; and assaying said third soybean plant for presence of DNA comprising one or more sequences selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof.

In another embodiment the invention provides an isolated DNA molecule that is diagnostic for soybean event 9582.814.19.1. Such molecules include, in addition to SEQ ID NOS: 1 and 2, molecules at least 25 bp in length comprising bp 1400-1401 of SEQ ID NO:1 and at least 10 bp of SEQ ID NO:1 in each direction from the bp 1400/1401 junction; amplicons at least 25 bp in length comprising 152-153 of SEQ ID NO:2 and at least 10 bp of SEQ ID NO:2 in each direction from the bp 152/153 junction. Examples are bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof.

4

In another embodiment the invention provides a method of controlling pests in soybean grain, seed, or seed meal which comprises including soybean event 9582.814.19.1 in said grain, seed, or seed meal as demonstrated by said grain, seed, or seed meal comprising DNA comprising one or more sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof.

The invention also includes soybean plant cells and plant parts including, but are not limited to pollen, ovule, flowers, shoots, roots, and leaves, and nuclei of vegetative cells, pollen cells, seed and seed meal, and egg cells, that contain soybean event 9582.814.19.1.

In some embodiments, soybean event 9582.814.19.1 can be combined with other traits, including, for example, other herbicide tolerance gene(s) and/or insect-inhibitory proteins and transcription regulatory sequences (i.e. RNA interference, dsRNA, transcription factors, etc). The additional traits may be stacked into the plant genome via plant breeding, re-transformation of the transgenic plant containing soybean event 9582.814.19.1, or addition of new traits through targeted integration via homologous recombination.

Other embodiments include the excision of polynucleotide sequences which comprise soybean event 9582.814.19.1, including for example, the pat gene expression cassette. Upon excision of a polynucleotide sequence, the modified event may be re-targeted at a specific chromosomal site wherein additional polynucleotide sequences are stacked with soybean event 9582.814.19.1.

In one embodiment, the present invention encompasses a soybean chromosomal target site located on chromosome 02 between the flanking sequences set forth in SEQ ID NOS:1 and 2.

In one embodiment, the present invention encompasses a method of making a transgenic soybean plant comprising inserting a heterologous nucleic acid at a position on chromosome 02 between the genomic sequences set forth in SEQ ID NOS:1 and 2, i.e. between bp 1-1400 of SEQ ID NO:1 and bp 153-1550 of SEQ ID NO:2.

Additionally, the subject invention provides assays for detecting the presence of the subject event in a sample (of soybeans, for example). The assays can be based on the DNA sequence of the recombinant construct, inserted into the soybean genome, and on the genomic sequences flanking the insertion site. Kits and conditions useful in conducting the assays are also provided.

The subject invention relates in part to the cloning and analysis of the DNA sequences of the border regions resulting from insertion of T-DNA from pDAB9582 in transgenic soybean lines. These sequences are unique. Based on the insert and junction sequences, event-specific primers can be and were generated. PCR analysis demonstrated that these events can be identified by analysis of the PCR amplicons generated with these event-specific primer sets. Thus, these and other related procedures can be used to uniquely identify soybean lines comprising the event of the subject invention.

#### SEED DEPOSIT

As part of this disclosure at least 2500 seeds of a soybean line comprising soybean event 9582.814.19.1 were deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va., 20110. The deposit, ATCC Patent Deposit Designation, PTA-12006, was received by the ATCC on Jul. 21, 2011. This deposit was made and will

US 8,680,363 B2

5

be maintained in accordance with and under the terms of the Budapest Treaty with respect to seed deposits for the purposes of patent procedure. This deposit was made and will be maintained in accordance with and under the terms of the Budapest Treaty with respect to seed deposits for the purposes of patent procedure.

#### BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the 5' DNA flanking border sequence for soybean event 9582.814.19.1. Nucleotides 1-1400 are genomic sequence. Nucleotides 1401-1535 are a rearranged sequence from pDAB9582. Nucleotides 1536-1836 are insert sequence.

SEQ ID NO:2 is the 3' DNA flanking border sequence for soybean event 9582.814.19.1. Nucleotides 1-152 are insert sequence. Nucleotides 153-1550 are genomic sequence.

SEQ ID NO:3 is the DNA sequence of pDAB9582, which is annotated below in Table 1.

SEQ ID NO:4 is oligonucleotide primer 81419\_FW3 for confirmation of 5' border genomic DNA.

SEQ ID NO:5 is oligonucleotide primer 81419\_RV1 for confirmation of 3' border genomic DNA.

SEQ ID NO:6 is oligonucleotide primer 81419\_RV2 for confirmation of 3' border genomic DNA.

SEQ ID NO:7 is oligonucleotide primer 81419\_RV3 for confirmation of 3' border genomic DNA.

SEQ ID NO:8 is oligonucleotide primer 5'IREnd-01 for confirmation of 5' border genomic DNA.

SEQ ID NO:9 is oligonucleotide primer 5'IREnd-02 for confirmation of 5' border genomic DNA.

SEQ ID NO:10 is oligonucleotide primer AtUbi10RV1 for confirmation of 5' border genomic DNA.

SEQ ID NO:11 is oligonucleotide primer AtUbi10RV2 for confirmation of 5' border genomic DNA.

SEQ ID NO:12 is oligonucleotide primer 3'PATEnd05 for confirmation of 3' border genomic DNA.

SEQ ID NO:13 is oligonucleotide primer 3'PATEnd06 for confirmation of 3' border genomic DNA.

SEQ ID NO:14 is the confirmed sequence of soybean event 9582.814.19.1. Including the 5' genomic flanking sequence, pDAB9582 T-strand insert, and 3' genomic flanking sequence.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a plasmid Map of pDAB9582 containing the cry1F, cry1Ac and pat expression cassettes.

FIG. 2 depicts the primer locations for confirming the 5' and 3' border sequence of the soybean event pDAB9582.814.19.1.

FIG. 3 depicts the genomic sequence arrangement in soybean event pDAB9582.814.19.1

#### DETAILED DESCRIPTION OF THE INVENTION

Both ends of the soybean event 9582.814.19.1 insertion have been sequenced and characterized. Event specific assays were developed. It has also been mapped onto the soybean genome (soybean chromosome 02). The event can be introgressed into further elite lines.

As alluded to above in the Background section, the introduction and integration of a transgene into a plant genome involves some random events (hence the name "event" for a given insertion that is expressed). That is, with many transformation techniques such as *Agrobacterium* transformation, the biolistic transformation (i.e. gene gun), and silicon car-

6

bide mediated transformation (i.e. WHISKERS), it is unpredictable where in the genome a transgene will become inserted. Thus, identifying the flanking plant genomic DNA on both sides of the insert can be important for identifying a plant that has a given insertion event. For example, PCR primers can be designed that generate a PCR amplicon across the junction region of the insert and the host genome. This PCR amplicon can be used to identify a unique or distinct type of insertion event.

Definitions and examples are provided herein to help describe the present invention and to guide those of ordinary skill in the art to practice the invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. The nomenclature for DNA bases as set forth at 37 CFR §1.822 is used.

As used herein, the term "progeny" denotes the offspring of any generation of a parent plant which comprises soybean event 9582.814.19.1.

A transgenic "event" is produced by transformation of plant cells with heterologous DNA, i.e., a nucleic acid construct that includes the transgenes of interest, regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. The term "event" refers to the original transformant and progeny of the transformant that include the heterologous DNA. The term "event" also refers to progeny produced by a sexual outcross between the transformant and another variety that includes the genomic/transgene DNA. Even after repeated back-crossing to a recurrent parent, the inserted transgene DNA and flanking genomic DNA (genomic/transgene DNA) from the transformed parent is present in the progeny of the cross at the same chromosomal location. The term "event" also refers to DNA from the original transformant and progeny thereof comprising the inserted DNA and flanking genomic sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

A "junction sequence" or "border sequence" spans the point at which DNA inserted into the genome is linked to DNA from the soybean native genome flanking the insertion point, the identification or detection of one or the other junction sequences in a plant's genetic material being sufficient to be diagnostic for the event. Included are the DNA sequences that span the insertions in herein-described soybean events and similar lengths of flanking DNA. Specific examples of such diagnostic sequences are provided herein; however, other sequences that overlap the junctions of the insertions, or the junctions of the insertions and the genomic sequence, are also diagnostic and could be used according to the subject invention.

The subject invention relates in part to event identification using such flanking, junction, and insert sequences. Related PCR primers and amplicons are included in the invention. According to the subject invention, PCR analysis methods using amplicons that span across inserted DNA and its borders can be used to detect or identify commercialized transgenic soybean varieties or lines derived from the subject proprietary transgenic soybean lines.

The flanking/junction sequences are diagnostic for soybean event 9582.814.19.1. Based on these sequences, event-specific primers were generated. PCR analysis demonstrated

US 8,680,363 B2

7

that these soybean lines can be identified in different soybean genotypes by analysis of the PCR amplicons generated with these event-specific primer sets. Thus, these and other related procedures can be used to uniquely identify these soybean lines. The sequences identified herein are unique.

Detection techniques of the subject invention are especially useful in conjunction with plant breeding, to determine which progeny plants comprise a given event, after a parent plant comprising an event of interest is crossed with another plant line in an effort to impart one or more additional traits of interest in the progeny. These PCR analysis methods benefit soybean breeding programs as well as quality control, especially for commercialized transgenic soybean seeds. PCR detection kits for these transgenic soybean lines can also now be made and used. This can also benefit product registration and product stewardship.

Furthermore, flanking soybean/genomic sequences can be used to specifically identify the genomic location of each insert. This information can be used to make molecular marker systems specific to each event. These can be used for accelerated breeding strategies and to establish linkage data.

Still further, the flanking sequence information can be used to study and characterize transgene integration processes, genomic integration site characteristics, event sorting, stability of transgenes and their flanking sequences, and gene expression (especially related to gene silencing, transgene methylation patterns, position effects, and potential expression-related elements such as MARS [matrix attachment regions], and the like).

In light of all the subject disclosure, it should be clear that the subject invention includes seeds available under the ATCC Deposit No. identified in paragraph [0021]. The subject invention also includes a herbicide-tolerant soybean plant grown from a seed deposited with the ATCC Deposit No. identified in paragraph [0021]. The subject invention further includes parts of said plant, such as leaves, tissue samples, seeds produced by said plant, pollen, and the like (wherein they comprise cry1F, cry1Ac, pat, and SEQ ID NOS: 1 and 2).

Still further, the subject invention includes descendant and/or progeny plants of plants grown from the deposited seed, preferably a herbicide-resistant soybean plant wherein said plant has a genome comprising a detectable wild-type junction sequence as described herein. As used herein, the term "soybean" means *Glycine max* and includes all varieties thereof that can be bred with a soybean plant.

This invention further includes processes of making crosses using a plant of the subject invention as at least one parent. For example, the subject invention includes an F<sub>1</sub> hybrid plant having as one or both parents any of the plants exemplified herein. Also within the subject invention is seed produced by such F<sub>1</sub> hybrids of the subject invention. This invention includes a method for producing an F<sub>1</sub> hybrid seed by crossing an exemplified plant with a different (e.g. in-bred parent) plant and harvesting the resultant hybrid seed. The subject invention includes an exemplified plant that is either a female parent or a male parent. Characteristics of the resulting plants may be improved by careful consideration of the parent plants.

An insect resistant/glufosinate-tolerant soybean plant of the subject invention can be bred by first sexually crossing a first parental soybean plant consisting of a soybean plant grown from seed of any one of the lines referred to herein, and a second parental soybean plant, thereby producing a plurality of first progeny plants; then selecting a first progeny plant that is resistant to glufosinate; selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants a plant that is

8

resistant to glufosinate. These steps can further include the back-crossing of the first progeny plant or the second progeny plant to the second parental soybean plant or a third parental soybean plant. A soybean crop comprising soybean seeds of the subject invention, or progeny thereof, can then be planted.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating, added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Other breeding methods commonly used for different traits and crops are known in the art. Backcross breeding has been used to transfer genes for a simply inherited, highly heritable trait into a desirable homozygous cultivar or inbred line, which is the recurrent parent. The source of the trait to be transferred is called the donor parent. The resulting plant is expected to have the attributes of the recurrent parent (e.g., cultivar) and the desirable trait transferred from the donor parent. After the initial cross, individuals possessing the phenotype of the donor parent are selected and repeatedly crossed (backcrossed) to the recurrent parent. The resulting parent is expected to have the attributes of the recurrent parent (e.g., cultivar) and the desirable trait transferred from the donor parent.

Likewise an insect resistant/glufosinate-tolerant soybean plant of the subject invention can be transformed with additional transgenes using methods known in the art. Transformation techniques such as *Agrobacterium* transformation, the biolistic transformation (i.e. gene gun), and silicon carbide mediated transformation (i.e. WHISKERS), can be used to introduced additional transgene(s) into the genome of soybean event 9582.814.19.1. Selection and characterization of transgenic plants containing the newly inserted transgenes can be completed to identify plants which contain a stable integrant of the novel transgene in addition to cry1F, cry1Ac, pat genes of the subject invention.

The DNA molecules of the present invention can be used as molecular markers in a marker assisted breeding (MAB) method. DNA molecules of the present invention can be used in methods (such as, AFLP markers, RFLP markers, RAPD markers, SNPs, and SSRs) that identify genetically linked agronomically useful traits, as is known in the art. The insect resistance and herbicide-tolerance traits can be tracked in the progeny of a cross with a soybean plant of the subject invention (or progeny thereof and any other soybean cultivar or variety) using the MAB methods. The DNA molecules are markers for this trait, and MAB methods that are well known in the art can be used to track the herbicide-resistance trait(s) in soybean plants where at least one soybean line of the subject invention, or progeny thereof, was a parent or ancestor. The methods of the present invention can be used to identify any soybean variety having the subject event.

Methods of the subject invention include a method of producing an insect resistant/herbicide-tolerant soybean plant wherein said method comprises breeding with a plant of the subject invention. More specifically, said methods can comprise crossing two plants of the subject invention, or one plant of the subject invention and any other plant. Preferred methods further comprise selecting progeny of said cross by analyzing said progeny for an event detectable according to the subject invention and favorable varietal performance (e.g. yield). For example, the subject invention can be used to track the subject event through breeding cycles with plants comprising other desirable traits, such as agronomic traits, disease tolerance or resistance, nematode tolerance or resistance and

US 8,680,363 B2

9

maturity date. Plants comprising the subject event and the desired trait can be detected, identified, selected, and quickly used in further rounds of breeding, for example. The subject event/trait can also be combined through breeding, and tracked according to the subject invention, with further insect resistant trait(s) and/or with further herbicide tolerance traits. Embodiments of the latter are plants comprising the subject event combined with the aad-12 gene, which confers tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides, or with a gene encoding resistance to the herbicide dicamba.

Thus, the subject invention can be combined with, for example, traits encoding glyphosate resistance (e.g., resistant plant or bacterial EPSPS, GOX, GAT), glufosinate resistance (e.g., pat, bar), acetolactate synthase (ALS)-inhibiting herbicide resistance (e.g., imidazolinones [such as imazethapyr], sulfonylureas, triazolopyrimidine sulfonanilide, pyrimidinylthiobenzoates, and other chemistries [Csr1, SurA, et al.]), bromoxynil resistance (e.g., Bxn), resistance to inhibitors of HPPD (4-hydroxyphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (e.g., psbA), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (e.g., PPO-1), resistance to phenylurea herbicides (e.g., CYP76B1), dicamba-degrading enzymes (see, e.g., US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

Additionally, soybean event 9582.814.19.1 can be combined with one or more additional input (e.g., insect resistance, pathogen resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

Methods to integrate a polynucleotide sequence within a specific chromosomal site of a plant cell via homologous recombination have been described within the art. For instance, site specific integration as described in US Patent Application Publication No. 2009/011188 A1, herein incorporated by reference, describes the use of recombinases or integrases to mediate the introduction of a donor polynucleotide sequence into a chromosomal target. In addition, International Patent Application No. WO 2008/021207, herein incorporated by reference, describes zinc finger mediated-homologous recombination to integrate one or more donor polynucleotide sequences within specific locations of the genome. The use of recombinases such as FLP/FRT as described in U.S. Pat. No. 6,720,475, herein incorporated by reference, or CRE/LOX as described in U.S. Pat. No. 5,658,772, herein incorporated by reference, can be utilized to integrate a polynucleotide sequence into a specific chromosomal site. Finally the use of meganucleases for targeting donor polynucleotides into a specific chromosomal location was described in Puchta et al., PNAS USA 93 (1996) pp. 5055-5060).

Other methods for site specific integration within plant cells are generally known and applicable (Kumar et al., *Trends in Plant Sci.* 6(4) (2001) pp. 155-159). Furthermore, site-specific recombination systems which have been identified in several prokaryotic and lower eukaryotic organisms may be applied to use in plants. Examples of such systems include, but are not limited too; the R/RS recombinase system from the pSR1 plasmid of the yeast *Zygosaccharomyces*

10

*rouxii* (Araki et al. (1985) J. Mol. Biol. 182: 191-203), and the Gin/gix system of phage Mu (Maeser and Kahlmann (1991) Mol. Gen. Genet. 230: 170-176).

In some embodiments of the present invention, it can be desirable to integrate or stack a new transgene(s) in proximity to an existing transgenic event. The transgenic event can be considered a preferred genomic locus which was selected based on unique characteristics such as single insertion site, normal Mendelian segregation and stable expression, and a superior combination of efficacy, including herbicide tolerance and agronomic performance in and across multiple environmental locations. The newly integrated transgenes should maintain the transgene expression characteristics of the existing transformants. Moreover, the development of assays for the detection and confirmation of the newly integrated event would be overcome as the genomic flanking sequences and chromosomal location of the newly integrated event are already identified. Finally, the integration of a new transgene into a specific chromosomal location which is linked to an existing transgene would expedite the introgression of the transgenes into other genetic backgrounds by sexual outcrossing using conventional breeding methods.

In some embodiments of the present invention, it can be desirable to excise polynucleotide sequences from a transgenic event. For instance transgene excision as described in Provisional U.S. Patent Application No. 61/297,628, herein incorporated by reference, describes the use of zinc finger nucleases to remove a polynucleotide sequence, consisting of a gene expression cassette, from a chromosomally integrated transgenic event. The polynucleotide sequence which is removed can be a selectable marker. Upon excision and removal of a polynucleotide sequence the modified transgenic event can be retargeted by the insertion of a polynucleotide sequence. The excision of a polynucleotide sequence and subsequent retargeting of the modified transgenic event provides advantages such as re-use of a selectable marker or the ability to overcome unintended changes to the plant transcriptome which results from the expression of specific genes.

The subject invention discloses herein a specific site on chromosome 02 in the soybean genome that is excellent for insertion of heterologous nucleic acids. Thus, the subject invention provides methods to introduce heterologous nucleic acids of interest into this pre-established target site or in the vicinity of this target site. The subject invention also encompasses a soybean seed and/or a soybean plant comprising any heterologous nucleotide sequence inserted at the disclosed target site or in the general vicinity of such site. One option to accomplish such targeted integration is to excise and/or substitute a different insert in place of the pat expression cassette exemplified herein. In this general regard, targeted homologous recombination, for example and without limitation, can be used according to the subject invention.

As used herein gene, event or trait "stacking" is combining desired traits into one transgenic line. Plant breeders stack transgenic traits by making crosses between parents that each have a desired trait and then identifying offspring that have both of these desired traits. Another way to stack genes is by transferring two or more genes into the cell nucleus of a plant at the same time during transformation. Another way to stack genes is by re-transforming a transgenic plant with another gene of interest. For example, gene stacking can be used to combine two or more different traits, including for example, two or more different insect traits, insect resistance trait(s) and disease resistance trait(s), two or more herbicide resistance traits, and/or insect resistance trait(s) and herbicide



US 8,680,363 B2

11

resistant trait(s). The use of a selectable marker in addition to a gene of interest can also be considered gene stacking.

“Homologous recombination” refers to a reaction between any pair of nucleotide sequences having corresponding sites containing a similar nucleotide sequence through which the two nucleotide sequences can interact (recombine) to form a new, recombinant DNA sequence. The sites of similar nucleotide sequence are each referred to herein as a “homology sequence.” Generally, the frequency of homologous recombination increases as the length of the homology sequence increases. Thus, while homologous recombination can occur between two nucleotide sequences that are less than identical, the recombination frequency (or efficiency) declines as the divergence between the two sequences increases. Recombination may be accomplished using one homology sequence on each of the donor and target molecules, thereby generating a “single-crossover” recombination product. Alternatively, two homology sequences may be placed on each of the target and donor nucleotide sequences. Recombination between two homology sequences on the donor with two homology sequences on the target generates a “double-crossover” recombination product. If the homology sequences on the donor molecule flank a sequence that is to be manipulated (e.g., a sequence of interest), the double-crossover recombination with the target molecule will result in a recombination product wherein the sequence of interest replaces a DNA sequence that was originally between the homology sequences on the target molecule. The exchange of DNA sequence between the target and donor through a double-crossover recombination event is termed “sequence replacement.”

A preferred plant, or a seed, of the subject invention comprises in its genome operative cry1F v3, cry1Ac synpro and pat v6 nucleotide sequences, as identified herein, together with at least 20-500 or more contiguous flanking nucleotides on both sides of the insert, as identified herein. Unless indicated otherwise, reference to flanking sequences refers to those identified with respect to SEQ ID NOS: 1 and 2. All or part of these flanking sequences could be expected to be transferred to progeny that receives the inserted DNA as a result of a sexual cross of a parental line that includes the event.

The subject invention includes tissue cultures of regenerable cells of a plant of the subject invention. Also included is a plant regenerated from such tissue culture, particularly where said plant is capable of expressing all the morphological and physiological properties of an exemplified variety. Preferred plants of the subject invention have all the physiological and morphological characteristics of a plant grown from the deposited seed. This invention further comprises progeny of such seed and seed possessing the quality traits of interest.

As used herein, a “line” is a group of plants that display little or no genetic variation between individuals for at least one trait. Such lines may be created by several generations of self-pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques.

As used herein, the terms “cultivar” and “variety” are synonymous and refer to a line which is used for commercial production.

“Stability” or “stable” means that with respect to the given component, the component is maintained from generation to generation and, preferably, at least three generations.

“Commercial Utility” is defined as having good plant vigor and high fertility, such that the crop can be produced by farmers using conventional farming equipment, and the oil

12

with the described components can be extracted from the seed using conventional crushing and extraction equipment

“Agronomically elite” means that a line has desirable agronomic characteristics such as yield, maturity, disease resistance, and the like, in addition to the insect resistance and herbicide tolerance due to the subject event(s). Any and all of these agronomic characteristics and data points can be used to identify such plants, either as a point or at either end or both ends of a range of characteristics used to define such plants.

As one skilled in the art will recognize in light of this disclosure, preferred embodiments of detection kits, for example, can include probes and/or primers directed to and/or comprising “junction sequences” or “transition sequences” (where the soybean genomic flanking sequence meets the insert sequence). For example, this includes a polynucleotide probes, primers, and/or amplicons designed to identify one or both junction sequences (where the insert meets the flanking sequence), as indicated in the Table above. One common design is to have one primer that hybridizes in the flanking region, and one primer that hybridizes in the insert. Such primers are often each about at least ~15 residues in length. With this arrangement, the primers can be used to generate/amplify a detectable amplicon that indicates the presence of an event of the subject invention. These primers can be used to generate an amplicon that spans (and includes) a junction sequence as indicated above.

The primer(s) “touching down” in the flanking sequence is typically not designed to hybridize beyond about 1200 bases or so beyond the junction. Thus, typical flanking primers would be designed to comprise at least 15 residues of either strand within 1200 bases into the flanking sequences from the beginning of the insert. That is, primers comprising a sequence of an appropriate size from (or hybridizing to) base pairs 800 to 1400 of SEQ ID NO:14 and/or base pairs 13,897 to 14,497 of SEQ ID NO:14 are within the scope of the subject invention. Insert primers can likewise be designed anywhere on the, but base pairs 1400 to 2000 of SEQ ID NO:14 and/or base pairs 13,297 to 13,896 of SEQ ID NO:14, and can be used, for example, non-exclusively for such primer design.

One skilled in the art will also recognize that primers and probes can be designed to hybridize, under a range of standard hybridization and/or PCR conditions wherein the primer or probe is not perfectly complementary to the exemplified sequence. That is, some degree of mismatch can be tolerated. For an approximately 20 nucleotide primer, for example, typically one or two or so nucleotides do not need to bind with the opposite strand if the mismatched base is internal or on the end of the primer that is opposite the amplicon. Various appropriate hybridization conditions are provided below. Synthetic nucleotide analogs, such as inosine, can also be used in probes. Peptide nucleic acid (PNA) probes, as well as DNA and RNA probes, can also be used. What is important is that such probes and primers are diagnostic for (able to uniquely identify and distinguish) the presence of an event of the subject invention.

It should be noted that errors in PCR amplification can occur which might result in minor sequencing errors, for example. That is, unless otherwise indicated, the sequences listed herein were determined by generating long amplicons from soybean genomic DNAs, and then cloning and sequencing the amplicons. It is not unusual to find slight differences and minor discrepancies in sequences generated and determined in this manner, given the many rounds of amplification that are necessary to generate enough amplicon for sequencing from genomic DNAs. One skilled in the art should recognize and be put on notice that any adjustments needed due

US 8,680,363 B2

13

to these types of common sequencing errors or discrepancies are within the scope of the subject invention.

It should also be noted that it is not uncommon for some genomic sequence to be deleted, for example, when a sequence is inserted during the creation of an event. Thus, some differences can also appear between the subject flanking sequences and genomic sequences listed in GENBANK, for example.

Components of the DNA sequence "insert" are illustrated in the Figures and are discussed in more detail below in the Examples. The DNA polynucleotide sequences of these components, or fragments thereof, can be used as DNA primers or probes in the methods of the present invention.

In some embodiments of the invention, compositions and methods are provided for detecting the presence of the transgene/genomic insertion region, in plants and seeds and the like, from a soybean plant. DNA sequences are provided that comprise the subject 5' transgene/genomic insertion region junction sequence provided herein (between base pairs 800 to 1400 of SEQ ID NO:14), segments thereof, and complements of the exemplified sequences and any segments thereof. DNA sequences are provided that comprise the subject 3' transgene/genomic insertion region junction sequence provided herein (between base pairs 13,897 to 14,497 of SEQ ID NO:14), segments thereof, and complements of the exemplified sequences and any segments thereof. The insertion region junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the soybean cell flanking the insertion site. Such sequences can be diagnostic for the given event.

Based on these insert and border sequences, event-specific primers can be generated. PCR analysis demonstrated that soybean lines of the subject invention can be identified in different soybean genotypes by analysis of the PCR amplicons generated with these event-specific primer sets. These and other related procedures can be used to uniquely identify these soybean lines. Thus, PCR amplicons derived from such primer pairs are unique and can be used to identify these soybean lines.

In some embodiments, DNA sequences that comprise a contiguous fragment of the novel transgene/genomic insertion region are an aspect of this invention. Included are DNA sequences that comprise a sufficient length of polynucleotides of transgene insert sequence and a sufficient length of polynucleotides of soybean genomic sequence from one or more of the three aforementioned soybean plants and/or sequences that are useful as primer sequences for the production of an amplicon product diagnostic for one or more of these soybean plants.

Related embodiments pertain to DNA sequences that comprise at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more contiguous nucleotides of a transgene portion of a DNA sequence identified herein (such as SEQ ID NO:1 and segments thereof), or complements thereof, and a similar length of flanking soybean DNA sequence from these sequences, or complements thereof. Such sequences are useful as DNA primers in DNA amplification methods. The amplicons produced using these primers are diagnostic for any of the soybean events referred to herein. Therefore, the invention also includes the amplicons produced by such DNA primers and homologous primers.

This invention also includes methods of detecting the presence of DNA, in a sample, that corresponds to the soybean event referred to herein. Such methods can comprise: (a) contacting the sample comprising DNA with a primer set that, when used in a nucleic acid amplification reaction with DNA from at least one of these soybean events, produces an ampli-

14

con that is diagnostic for said event(s); (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon.

Further detection methods of the subject invention include a method of detecting the presence of a DNA, in a sample, corresponding to said event, wherein said method comprises: (a) contacting the sample comprising DNA with a probe that hybridizes under stringent hybridization conditions with DNA from at least one of said soybean events and which does not hybridize under the stringent hybridization conditions with a control soybean plant (non-event-of-interest DNA); (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.

In still further embodiments, the subject invention includes methods of producing a soybean plant comprising soybean event 9582.814.19.1 of the subject invention, wherein said method comprises the steps of: (a) sexually crossing a first parental soybean line (comprising an expression cassettes of the present invention, which confers glufosinate tolerance to plants of said line) and a second parental soybean line (that lacks this herbicide tolerance trait) thereby producing a plurality of progeny plants; and (b) selecting a progeny plant by the use of molecular markers. Such methods may optionally comprise the further step of back-crossing the progeny plant to the second parental soybean line to producing a true-breeding soybean plant that comprises the insect resistant and glufosinate tolerant trait.

According to another aspect of the invention, methods of determining the zygosity of progeny of a cross with said event is provided. Said methods can comprise contacting a sample, comprising soybean DNA, with a primer set of the subject invention. Said primers, when used in a nucleic-acid amplification reaction with genomic DNA from at least one of said soybean events, produces a first amplicon that is diagnostic for at least one of said soybean events. Such methods further comprise performing a nucleic acid amplification reaction, thereby producing the first amplicon; detecting the first amplicon; and contacting the sample comprising soybean DNA with a second primer set (said second primer set, when used in a nucleic-acid amplification reaction with genomic DNA from soybean plants, produces a second amplicon comprising the native soybean genomic DNA homologous to the soybean genomic region); and performing a nucleic acid amplification reaction, thereby producing the second amplicon. The methods further comprise detecting the second amplicon, and comparing the first and second amplicons in a sample, wherein the presence of both amplicons indicates that the sample is heterozygous for the transgene insertion.

DNA detection kits can be developed using the compositions disclosed herein and methods well known in the art of DNA detection. The kits are useful for identification of the subject soybean event DNA in a sample and can be applied to methods for breeding soybean plants containing this DNA. The kits contain DNA sequences homologous or complementary to the amplicons, for example, disclosed herein, or to DNA sequences homologous or complementary to DNA contained in the transgene genetic elements of the subject events. These DNA sequences can be used in DNA amplification reactions or as probes in a DNA hybridization method. The kits may also contain the reagents and materials necessary for the performance of the detection method.

A "probe" is an isolated nucleic acid molecule to which is attached a conventional detectable label or reporter molecule (such as a radioactive isotope, ligand, chemiluminescent agent, or enzyme). Such a probe is complementary to a strand of a target nucleic acid, in the case of the present invention, to



US 8,680,363 B2

15

a strand of genomic DNA from one of said soybean events, whether from a soybean plant or from a sample that includes DNA from the event. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

"Primers" are isolated/synthesized nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs of the present invention refer to their use for amplification of a target nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

Probes and primers are generally 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, 500, or 1000, or 2000, or 5000 polynucleotides or more in length. Such probes and primers hybridize specifically to a target sequence under high stringency hybridization conditions. Preferably, probes and primers according to the present invention have complete sequence similarity with the target sequence, although probes differing from the target sequence and that retain the ability to hybridize to target sequences may be designed by conventional methods.

Methods for preparing and using probes and primers are described, for example, in *Molecular Cloning: A Laboratory Manual*, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring

16

Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989. PCR-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose.

Primers and probes based on the flanking DNA and insert sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed sequences by conventional methods, e.g., by re-cloning and sequencing such sequences.

The nucleic acid probes and primers of the present invention hybridize under stringent conditions to a target DNA sequence. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. Nucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook et al., 1989. Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

As used herein, a substantially homologous sequence is a nucleic acid sequence that will specifically hybridize to the complement of the nucleic acid sequence to which it is being compared under high stringency conditions. The term "stringent conditions" is functionally defined with regard to the hybridization of a nucleic-acid probe to a target nucleic acid (i.e., to a particular nucleic-acid sequence of interest) by the specific hybridization procedure discussed in Sambrook et al., 1989, at 9.52-9.55. See also, Sambrook et al., 1989 at 9.47-9.52 and 9.56-9.58. Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNA fragments.

Depending on the application envisioned, one can use varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically employ relatively stringent conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50° C. to about 70° C. Stringent conditions, for example, could involve washing the hybridization filter at least twice with high-stringency wash buffer (0.2×SSC, 0.1% SDS, 65° C.). Appropriate stringency conditions which promote DNA hybridization, for example, 6.0× sodium chloride/sodium citrate (SSC) at about 45° C.,

US 8,680,363 B2

17

followed by a wash of 2.0×SSC at 50° C. are known to those skilled in the art. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0×SSC at 50° C. to a high stringency of about 0.2×SSC at 50° C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22° C., to high stringency conditions at about 65° C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand. Detection of DNA sequences via hybridization is well-known to those of skill in the art, and the teachings of U.S. Pat. Nos. 4,965,188 and 5,176,995 are exemplary of the methods of hybridization analyses.

In a particularly preferred embodiment, a nucleic acid of the present invention will specifically hybridize to one or more of the primers (or amplicons or other sequences) exemplified or suggested herein, including complements and fragments thereof, under high stringency conditions. In one aspect of the present invention, a marker nucleic acid molecule of the present invention has the nucleic acid sequence as set forth herein in one of the exemplified sequences, or complements and/or fragments thereof.

In another aspect of the present invention, a marker nucleic acid molecule of the present invention shares between 80% and 100% or 90% and 100% sequence identity with such nucleic acid sequences. In a further aspect of the present invention, a marker nucleic acid molecule of the present invention shares between 95% and 100% sequence identity with such sequence. Such sequences may be used as markers in plant breeding methods to identify the progeny of genetic crosses. The hybridization of the probe to the target DNA molecule can be detected by any number of methods known to those skilled in the art, these can include, but are not limited to, fluorescent tags, radioactive tags, antibody based tags, and chemiluminescent tags.

Regarding the amplification of a target nucleic acid sequence (e.g., by PCR) using a particular amplification primer pair, “stringent conditions” are conditions that permit the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product, the amplicon.

The term “specific for (a target sequence)” indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

As used herein, “amplified DNA” or “amplicon” refers to the product of nucleic-acid amplification of a target nucleic acid sequence that is part of a nucleic acid template. For example, to determine whether the soybean plant resulting from a sexual cross contains transgenic event genomic DNA from the soybean plant of the present invention, DNA extracted from a soybean plant tissue sample may be subjected to nucleic acid amplification method using a primer pair that includes a primer derived from flanking sequence in the genome of the plant adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA. The amplicon is of a length and has a sequence that is also diagnostic for the event. The amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair, and/or the combined length of the primer pairs plus about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36,

18

37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, or 500, 750, 1000, 1250, 1500, 1750, 2000, or more nucleotide base pairs (plus or minus any of the increments listed above). Alternatively, a primer pair can be derived from flanking sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence. A member of a primer pair derived from the plant genomic sequence may be located a distance from the inserted DNA sequence. This distance can range from one nucleotide base pair up to about twenty thousand nucleotide base pairs. The use of the term “amplicon” specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

Nucleic-acid amplification can be accomplished by any of the various nucleic-acid amplification methods known in the art, including the polymerase chain reaction (PCR). A variety of amplification methods are known in the art and are described, inter alia, in U.S. Pat. No. 4,683,195 and U.S. Pat. No. 4,683,202. PCR amplification methods have been developed to amplify up to 22 kb of genomic DNA. These methods as well as other methods known in the art of DNA amplification may be used in the practice of the present invention. The sequence of the heterologous transgene DNA insert or flanking genomic sequence from a subject soybean event can be verified (and corrected if necessary) by amplifying such sequences from the event using primers derived from the sequences provided herein followed by standard DNA sequencing of the PCR amplicon or of the cloned DNA.

The amplicon produced by these methods may be detected by a plurality of techniques. Agarose gel electrophoresis and staining with ethidium bromide is a common well known

US 8,680,363 B2

19

method of detecting DNA amplicons. Another such method is Genetic Bit Analysis where an DNA oligonucleotide is designed which overlaps both the adjacent flanking genomic DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking genomic sequence), a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Another method is the Pyrosequencing technique as described by Winge (Innov. Pharma. Tech. 00:18-24, 2000). In this method an oligonucleotide is designed that overlaps the adjacent genomic DNA and insert DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking genomic sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. DNTPs are added individually and the incorporation results in a light signal that is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Fluorescence Polarization is another method that can be used to detect an amplicon of the present invention. Following this method, an oligonucleotide is designed which overlaps the genomic flanking and inserted DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking genomic DNA sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single base extension.

TAQMAN® (PE Applied Biosystems, Foster City, Calif.) is a method of detecting and quantifying the presence of a DNA sequence. Briefly, a FRET oligonucleotide probe is designed that overlaps the genomic flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. During specific amplification, Taq DNA polymerase cleaves and releases the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

Molecular Beacons have been described for use in sequence detection. Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking genomic and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal

20

indicates the presence of the flanking genomic/transgene insert sequence due to successful amplification and hybridization.

Having disclosed a location in the soybean genome that is excellent for an insertion, the subject invention also comprises a soybean seed and/or a soybean plant comprising at least one non-soybean event 9582.814.19.1 insert in the general vicinity of this genomic location. One option is to substitute a different insert in place of the one from soybean event pDAB9582.814.19.1 exemplified herein. In these general regards, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (US 20030232410). Thus, the subject invention includes plants and plant cells comprising a heterologous insert (in place of or with multi-copies of the cry1F, cry1Ac, or pat genes), flanked by all or a recognizable part of the flanking sequences identified herein (bp 1-1400 of SEQ ID NO:1 and bp 153-1550 of SEQ ID NO:2). An additional copy (or additional copies) of a cry1F, cry1Ac, or pat could also be targeted for insertion in this/these manner(s).

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification

The following examples are included to illustrate procedures for practicing the invention and to demonstrate certain preferred embodiments of the invention. These examples should not be construed as limiting. It should be appreciated by those of skill in the art that the techniques disclosed in the following examples represent specific approaches used to illustrate preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in these specific embodiments while still obtaining like or similar results without departing from the spirit and scope of the invention. Unless otherwise indicated, all percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

The following abbreviations are used unless otherwise indicated.

bp base pair  
° C. degrees Celsius  
DNA deoxyribonucleic acid  
EDTA ethylenediaminetetraacetic acid  
kb kilobase  
µg microgram  
µL microliter  
mL milliliter  
M molar mass  
PCR polymerase chain reaction  
PTU plant transcription unit  
SDS sodium dodecyl sulfate  
SSC a buffer solution containing a mixture of sodium chloride and sodium citrate, pH 7.0  
TBE a buffer solution containing a mixture of Tris base, boric acid and EDTA, pH 8.3

Embodiments of the present invention are further defined in the following Examples. It should be understood that these Examples are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the embodiments of the invention to adapt it to various usages and conditions. Thus, various modifications of the embodiments of the inven-

US 8,680,363 B2

21

tion, in addition to those shown and described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

## EXAMPLES

## Example 1

## Transformation and Selection of the Cry1F and Cry1Ac Soybean Event pDAB9582.814.19.1

Transgenic soybean (*Glycine max*) containing the soybean event pDAB9582.814.19.1 was generated through *Agrobacterium*-mediated transformation of soybean cotyledonary node explants. The disarmed *Agrobacterium* strain EHA101 (Hood et al., 1993), carrying the binary vector pDAB9582 (FIG. 1) containing the selectable marker, *pat* v6, and the genes of interest, *cry1F* v3 and *cry1Ac* synpro, within the T-strand DNA region, was used to initiate transformation. The DNA sequence for pDAB9582 is given in SEQ ID NO:3, which is annotated below in Table 1.

TABLE 1

Gene elements located on pDAB9582.		
bp (SEQ ID NO: 3)	Construct element	Reference
272-1593	AtUbi10 Promoter	Callis, et al., (1990) <i>J. Biol. Chem.</i> , 265: 12486-12493
1602-5048	Cry1F	Referenced above
5151-5607	ORF23 3'UTR	U.S. Pat. No. 5,428,147
5671-6187	CsVMV Promoter	Verdaguer et al., (1996) <i>Plant Mol. Biol.</i> , 31: 1129-1139
6197-9667	Cry 1AC	Referenced above
9701-10157	ORF23 3'UTR	U.S. Pat. No. 5,428,147
10272-10788	CsVMV Promoter	Verdaguer et al., (1996) <i>Plant Mol. Biol.</i> , 31: 1129-1139
10796-11347	PAT	Wohlleben et al., (1988) <i>Gene</i> 70: 25-37
11450-12153	ORF1 3'UTR	Huang et al., (1990) <i>J. Bacteriol.</i> 172: 1814-1822

*Agrobacterium*-mediated transformation was carried out using a modified procedure of Zeng et al. (2004). Briefly, soybean seeds (cv Maverick) were germinated on basal media and cotyledonary nodes were isolated and infected with *Agrobacterium*. Shoot initiation, shoot elongation, and rooting media were supplemented with cefotaxime, timentin and vancomycin for removal of *Agrobacterium*. Glufosinate selection was employed to inhibit the growth of non-transformed shoots. Selected shoots were transferred to rooting medium for root development and then transferred to soil mix for acclimatization of plantlets.

Terminal leaflets of selected plantlets were leaf painted with glufosinate to screen for putative transformants. The screened plantlets were transferred to the greenhouse, allowed to acclimate and then leaf-painted with glufosinate to reconfirm tolerance and deemed to be putative transformants. The screened plants were sampled and molecular analyses for the confirmation of the selectable marker gene and/or the gene of interest were carried out. T<sub>0</sub> plants were allowed to self fertilize in the greenhouse to give rise to T<sub>1</sub> seed.

This event, soybean event pDAB9582.814.19.1, was generated from an independent transformed isolate. The T<sub>1</sub> plants were backcrossed and introgressed into elite varieties over subsequent generations. The event was selected based on its

22

unique characteristics such as single insertion site, normal Mendelian segregation, stable expression, and a superior combination of efficacy, including herbicide tolerance and agronomic performance. The following examples contain the data which were used to characterize soybean event pDAB9582.814.19.1.

## Example 2

## Characterization of Protein Expression in Soybean Event pDAB9582.814.19.1

The biochemical properties of the recombinant Cry1F, Cry1Ac, and PAT proteins expressed in soybean event 9582.814.19.1 were characterized. Quantitative enzyme-linked immunosorbent assay (ELISA) is a biochemical assay known within the art that can be used to characterize the biochemical properties of the proteins and confirm expression of these proteins in soybean event 9582.814.19.1.

## Example 2.1

## Expression of the PAT, Cry1F, and Cry1Ac Protein in Plant Tissues

Samples of soybean tissues were isolated from the test plants and prepared for expression analysis. The PAT protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST) containing 0.5% Bovine Serum Albumin (BSA). The plant tissue was centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer as necessary, and analyzed using an PAT ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Envirologix, Portland, Me.). This assay measured the expressed PAT protein.

The Cry1F protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST). The plant tissue was centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer as necessary, and analyzed using an Cry1F ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Strategic Diagnostics Inc., Newark, Del.). This assay measured the expressed Cry1F protein.

The Cry1Ac protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST) containing 0.5% Bovine Serum Albumin (BSA). The plant tissue was centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer as necessary, and analyzed using an Cry1Ac ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Strategic Diagnostics Inc., Newark, Del.). This assay measured the Cry1Ac protein.

Detection analysis was performed to investigate the expression stability and inheritability both vertically (between generations) and horizontally (between lineages within a generation) in soybean event pDAB9582.814.19.1.

## Example 2.2

## Expression of the PAT, Cry1F, and Cry1Ac Protein in Plant Tissues

Levels of Cry1F, Cry1Ac and PAT proteins were determined in Soybean Event 9582.814.19.1. The soluble, extractable proteins were measured using a quantitative enzyme-



## US 8,680,363 B2

23

linked immunosorbent assay (ELISA) method from soybean leaf tissue. From T<sub>2</sub> to T<sub>6</sub> generations Soybean Events 9582.814.19.1, expression was stable (not segregating) and consistent across all lineages. Table 2 lists the mean expression level of the transgenic proteins in soybean event 9582.814.19.1.

TABLE 2

Mean expression level of different transgenic proteins in soybean event pDAB9582.814.19.1.			
Expression Level of Different Proteins (ng/cm <sup>2</sup> )			
Event	Cry1F	Cry1 Ac	PAT
Soybean event pDAB9582.814.19.1	133	17.4	12

## Example 3

## Cloning and Characterization of DNA Sequence in the Insert and the Flanking Border Regions of Soybean Event pDAB9582.814.19.1

To characterize and describe the genomic insertion site, the sequence of the flanking genomic T-DNA border regions of

24

soybean event pDAB9582.814.19.1 were determined. Genomic sequence of soybean event pDAB9582.814.19.1 was confirmed, comprising 1400 bp of 5' flanking border sequence (SEQ ID NO:1) and 1398 bp of 3' flanking border sequence (SEQ ID NO:2). PCR amplification based on the soybean event pDAB9582.814.19.1 border sequences validated that the border regions were of soybean origin and that the junction regions are unique sequences for soybean event pDAB9582.814.19.1. The junction regions could be used for event-specific identification of soybean event pDAB9582.814.19.1. In addition, the T-strand insertion site was characterized by amplifying a genomic fragment corresponding to the region of the identified flanking border sequences from the genome of untransformed soybean. Comparison of soybean event pDAB9582.814.19.1 with the untransformed genomic sequence revealed that a deletion of about 57 bp from the original locus resulted during the T-strand integration. Overall, the characterization of the insert and border sequence of soybean event pDAB9582.814.19.1 indicated that an intact copy of the T-strand from pDAB9582 was present in the soybean genome.

TABLE 3

List of primers and their sequences used in the confirmation of soybean genomic DNA in soybean event pDAB9582.814.19.1				
SEQ ID NO:	Primer Name	Size (bp)	Sequence (5' to 3')	Purpose
SEQ ID NO: 4	81419_FW3	30	TTTCTCCTATCCGTC AAATAAATCTGCTCC	confirmation of 5' border genomic DNA, used with AtUbi10RV1 or RV2; with 5'IREnd-01 or 5'IREnd-02
SEQ ID NO: 5	81419_RV1	27	GGTGATTTGGTGCC AAAAGTTATGTT	confirmation of 3' border genomic DNA, used with 3'PATEnd05 or 3'PATEnd06
SEQ ID NO: 6	81419_RV2	24	TGGAGGGTCATATCG CAAAAGACT	confirmation of 3' border genomic DNA, used with 3'PATEnd05 or 3'PATEnd06
SEQ ID NO: 7	81419_RV3	24	GTTCTGCGTCGTGGA GGTTCATAT	confirmation of 3' border genomic DNA, used with 3'PATEnd05 or 3'PATEnd06
SEQ ID NO: 8	5'IREnd-01	29	CGAGCTTTCTAATTT CAAACATTCGGGC	confirmation of 5' border genomic DNA, used with 81419_FW3
SEQ ID NO: 9	5'IREnd-02	30	TCCTAGATCATCAGT TCATACAAACCTCCA	confirmation of 5' border genomic DNA, used with 81419_FW3
SEQ ID NO: 10	AtUbi10RV1	29	CGGTCCTAGATCATC AGTTCATACAAACC	confirmation of 5' border genomic DNA, used with 81419_FW3
SEQ ID NO: 11	AtUbi10RV2	28	CACTCGTGTTCAGTC CAATGACCAATAA	confirmation of 5' border genomic DNA, used with 81419_FW3
SEQ ID NO: 12	3'PATEnd05	20	GCTCCTCCAAGGCCA GTTAG	confirmation of 3' border genomic DNA, used with 81419_RV1, RV2 or RV3
SEQ ID NO: 13	3'PATEnd06	20	CCAGTTAGGCCAGTT ACCCA	confirmation of 3' border genomic DNA, used with 81419_RV1, RV2 or RV3

US 8,680,363 B2

25

26

TABLE 4

Conditions for standard PCR amplification of the border regions and event-specific sequences in soybean event pDAB9582.814.19.1.						
Target Sequence	Primer Set	PCR Mixture	Pre-denaturation (° C./min)	Denaturation (° C./sec.)	Extension (° C./min:sec)	Final Extension (° C./min)
5' border	81419_FW3/ AtUbi10RV1	D	95/3	98/10	68/4:00 32 cycles	72/10
5' border	81419_FW3/ 5'TREnd-01	D	95/3	98/10	68/4:00 32 cycles	72/10
3' border	3'PATEnd05/ 81419_RV2	D	95/3	98/10	68/4:00 35 cycles	72/10
3' border	3'PATEnd05/ 81419_RV3	D	95/3	98/10	68/4:00 35 cycles	72/10
3' border	3'PATEnd06/ 81419_RV2	D	95/3	98/10	68/4:00 35 cycles	72/10
3' border	3'PATEnd06/ 81419_RV3	D	95/3	98/10	68/4:00 32 cycles	72/10
Across the insert locus	81419_FW3/ 81419_RV3	D	95/3	98/10	68/4:00 32 cycles	72/10

TABLE 5

PCR mixture for standard PCR amplification of the border regions and event specific sequences in soybean event pDAB9582.814.19.1.			
Reagent	1 x reaction (μL)	Reagent	1 x reaction (μL)
PCR Mixture A		PCR Mixture B	
H2O	0.8	H2O	14.6
ACCPRIIME PFX SUPERMIX	20	10X LA TAQ	2
—	—	BUFFER	—
—	—	MgCl <sub>2</sub> (25 mM)	0.6
—	—	dNTP (2.5 uM)	1.6
10 uM primer	0.2	10 uM primer	0.1
gDNA digestion	1	gDNA digestion	1
—	—	LA TAQ (5U/ul)	0.1
rxn vol:	22	rxn vol:	20
PCR Mixture C		PCR Mixture D	
H2O	28	H2O	11.6
10X PCR buffer II (Mg-plus)	5	10X PCR buffer II (Mg-plus)	2
MgCl <sub>2</sub> [25 mM]	1.5	MgCl <sub>2</sub> [25 mM]	0.6
dNTP [2.5 mM]	8	dNTP [2.5 mM]	3.2
Adaptor PCR primer (10 μM)	1	primer1 (10 μM)	0.4
GOI nested primer (10 μM)	1	primer2 (10 μM)	0.4
DNA binded Beads	5	DNA Template	0.2
LA TAQ (5U/ul)	0.5	LA TAQ (5U/ul)	1.6
rxn vol:	50	rxn vol:	20

## Example 3.1

## Confirmation of Soybean Genomic Sequences

The 5' and 3' flanking borders aligned to a *Glycine max* whole genome shotgun sequence from chromosome 02, indicating that the transgene of soybean event pDAB9582.814.19.1 was inserted in soybean genome chromosome 02. To confirm the insertion site of soybean event pDAB9582.814.19.1 from the soybean genome, PCR was carried out with different pairs of primers (FIG. 2, Table 3, Table 4, and Table 5). Genomic DNA from soybean event pDAB9582.814.19.1 and other transgenic or non-transgenic soybean lines was used as a template. To confirm that the 5' border sequences are correct a primer designed to bind to the At Ubi10 promoter gene element, for example AtUbi10RV1, and a primer designed to bind to the cloned 5' end border on soybean genome chromosome 02, primer designated

81419\_FW3, were used for amplifying the DNA segment that spans the At Ubi10 promoter gene element to 5' end border sequence. Similarly, for confirmation of the cloned 3' border sequence a pat specific primer, for example 3'PATEnd05, and three primers designed according to the cloned 3' end border sequence, designated 81419\_RV1, 81419\_RV2 and 81419\_RV3, were used for amplifying DNA segments that span the pat gene to 3' border sequence. DNA fragments with expected sizes were amplified only from the genomic DNA of soybean event pDAB9582.814.19.1 with each primer pair, but not from DNA samples from other transgenic soybean lines or the non-transgenic control. The results indicate that the cloned 5' and 3' border sequences are the flanking border sequences of the T-strand insert for soybean event pDAB9582.814.19.1.

To further confirm the DNA insertion in the soybean genome, a PCR amplification spanning the soybean border sequences was completed on genomic DNA which did not



US 8,680,363 B2

27

contain the T-strand insert for soybean event pDAB9582.814.19.1. Primer 81419\_FW3, designed according to the 5' end border sequence, and one primer 81419-RV3, designed for the 3' end border sequence, were used to amplify DNA segments which contained the locus where the pDAB9582 T-strand integrated. As expected, PCR amplification completed with the primer pair of 81419\_FW3 and 81419\_RV3 produced an approximately a 1.5 kb DNA fragment from all the other soybean control lines but not pDAB9582.814.19.1. Aligning the identified 5' and 3' border sequences of soybean event pDAB9582.814.19.1 with a *Glycine max* whole genome shotgun sequence from chromosome 02 revealed about 57 bp deletion from the original locus. (FIG. 3). These results demonstrated that the transgene of soybean event pDAB8294 was inserted into the site of soybean genome chromosome 02.

## Example 4

Soybean Event pDAB9582.814.19.1  
Characterization via Southern Blot

Southern blot analysis was used to establish the integration pattern of soybean event pDAB9582.814.19.1. These experiments generated data which demonstrated the integration and integrity of the cry1Ac and cry1F transgenes within the soybean genome. Soybean event pDAB9582.814.19.1 was characterized as a full length, simple integration event containing a single copy of the cry1Ac and cry1F plant transcription unit (PTU) from plasmid pDAB9582.

Southern blot data suggested that a T-strand fragment inserted into the genome of soybean event pDAB9582.814.19.1. Detailed Southern blot analysis was conducted using probes specific to the cry1Ac and cry1F gene, contained in the T-strand integration region of pDAB9582.814.19.1, and descriptive restriction enzymes that have cleavage sites located within the plasmid and produce hybridizing fragments internal to the plasmid or fragments that span the junction of the plasmid with soybean genomic DNA (border fragments). The molecular weights indicated from the Southern hybridization for the combination of the restriction enzyme and the probe were unique for the event, and established its identification patterns. These analyses also showed that the plasmid fragment had been inserted into soybean genomic DNA without rearrangements of the cry1Ac and cry1F PTU.

## Example 4.1

Soybean Leaf Sample Collection and Genomic DNA  
(gDNA) Isolation

Genomic DNA was extracted from leaf tissue harvested from individual soybean plants containing soybean event pDAB9582.814.19.1. In addition, gDNA was isolated from a conventional soybean plant, Maverick, which contains the genetic background that is representative of the substance line, absent the cry1Ac and cry1F genes. Individual genomic DNA was extracted from lyophilized leaf tissue following the standard CTAB method (Sambrook et al (1989)). Following extraction, the DNA was quantified spectrofluorometrically using PICO GREEN reagent (Invitrogen, Carlsbad, Calif.). The DNA was then visualized on an agarose gel to confirm values from the PICO GREEN analysis and to determine the DNA quality.

28

## Example 4.2

## DNA Digestion and Separation

For Southern blot molecular characterization of soybean event pDAB9582.814.19.1, ten micrograms (10 µg) of genomic DNA was digested. Genomic DNA from the soybean event pDAB9582.814.19.1 and non-transgenic soybean line Maverick was digested by adding approximately five units of selected restriction enzyme per µg of DNA and the corresponding reaction buffer to each DNA sample. Each sample was incubated at approximately 37° C. overnight. The restriction enzymes AseI, HindIII, NsiI, and NdeI were used individually for the single digests (New England Biolabs, Ipswich, Mass.). The restriction enzymes NotI and ApaI were used together for a double digestion (New England Biolabs, Ipswich, Mass.). In addition, a positive hybridization control sample was prepared by combining plasmid DNA, pDAB9582 with genomic DNA from the non-transgenic soybean variety, Maverick. The plasmid DNA/genomic DNA cocktail was digested using the same procedures and restriction enzyme as the test samples.

After the digestions were incubated overnight, 25 µL QUICK-PRECIP PLUS SOLUTION (Edge Biosystems, Gaithersburg, Md.) was added and the digested DNA samples were precipitated with isopropanol. The precipitated DNA pellet was resuspended in 15 µL of 1× loading buffer (0.01% bromophenol blue, 10.0 mM EDTA, 10.0% glycerol, 1.0 mM Tris pH 7.5). The DNA samples and molecular size markers were then electrophoresed through 0.85% agarose gels with 0.4× TAE buffer (Fisher Scientific, Pittsburgh, Pa.) at 35 volts for approximately 18-22 hours to achieve fragment separation. The gels were stained with ethidium bromide (Invitrogen, Carlsbad, Calif.) and the DNA was visualized under ultraviolet (UV) light.

## Example 4.3

## Southern Transfer and Membrane Treatment

Southern blot analysis was performed essentially as described by Memelink, et al. (1994). Briefly, following electrophoretic separation and visualization of the DNA fragments, the gels were depurinated with 0.25M HCl for approximately 20 minutes, and then exposed to a denaturing solution (0.4 M NaOH, 1.5 M NaCl) for approximately 30 minutes followed by neutralizing solution (1.5 M NaCl, 0.5 M Tris pH 7.5) for at least 30 minutes. Southern transfer was performed overnight onto nylon membranes using a wicking system with 10×SSC. After transfer the DNA was bound to the membrane by UV crosslinking following by briefly washing membrane with a 2×SSC solution. This process produced Southern blot membranes ready for hybridization.

## Example 4.4

## DNA Probe Labeling and Hybridization

The DNA fragments bound to the nylon membrane were detected using a labeled probe (Table 6). Probes were generated by a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, into the DNA fragment amplified from plasmid pDAB9582 using primers specific to gene elements. Generation of DNA probes by PCR synthesis was carried out using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Indianapolis, Ind.) following the manufacturer's recommended procedures.

US 8,680,363 B2

29

Labeled probes were analyzed by agarose gel electrophoresis to determine their quality and quantity. A desired amount of labeled probe was then used for hybridization to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG EASY HYB SOLUTION (Roche Diagnostics, Indianapolis, Ind.). Briefly, nylon membrane blots containing fixed DNA were briefly washed with 2×SSC and pre-hybridized with 20-25 mL of pre-warmed DIG EASY HYB SOLUTION in hybridization bottles at approximately 45-55° C. for about 2 hours in a hybridization oven. The pre-hybridization solution was then decanted and replaced with ~15 mL of pre-warmed DIG EASY HYB SOLUTION containing a desired amount of specific probes denatured by boiling in a water bath for approximately five minutes. The hybridization step was then conducted at approximately 45-55° C. overnight in the hybridization oven.

At the end of the probe hybridization, DIG EASY HYB SOLUTIONS containing the probes were decanted into clean tubes and stored at approximately -20° C. These probes could be reused up to two times according to the manufacturer's recommended procedure. The membrane blots were rinsed briefly and washed twice in clean plastic containers with low stringency wash buffer (2×SSC, 0.1% SDS) for approximately five minutes at room temperature, followed by washing twice with high stringency wash buffer (0.1×SSC, 0.1% SDS) for 15 minutes each at approximately 65° C. The membrane blots briefly washed with 1× Maleic acid buffer from the DIG WASH AND BLOCK BUFFER SET (Roche Diagnostics, Indianapolis, Ind.) for approximately 5 minutes. This was followed by blocking in a 1× blocking buffer for 2 hours and an incubation with anti-DIG-AP (alkaline phosphatase) antibody (Roche Diagnostics, Indianapolis, Ind.) in 1× blocking buffer also for a minimum of 30 minutes. After 2-3 washes with 1× washing buffer, specific DNA probes remain bound to the membrane blots and DIG-labeled DNA standards were visualized using CDP-STAR CHEMILUMINESCENT NUCLEIC ACID DETECTION SYSTEM (Roche Diagnostics, Indianapolis, Ind.) following the manufacturer's recommendation. Blots were exposed to chemiluminescent film for one or more time points to detect hybridizing fragments and to visualize molecular size standards. Films were developed with an ALL-PRO 100 PLUS film developer (Konica Minolta, Osaka, Japan) and images were scanned. The number and sizes of detected bands were documented for each probe. DIG-LABELED DNA MOLECULAR WEIGHT MARKER II (DIG MWM II) and DIG-LABELED DNA MOLECULAR WEIGHT MARKER VII (DIG MWM VII), visible after DIG detection as described, were used to determine hybridizing fragment size on the Southern blots.

TABLE 6

Location and length of probes used in Southern analysis.		
Probe Name	Genetic Element	Length (bp)
Cry1Ac	cry1Ac	1720
Cry1F	cry1F	1746
specR	Spectinomycin resistance gene	750
OriRep	Ori Rep	852
trfA	Replication initiation protein trfA	1119

## Example 4.5

## Southern Blot Results

Expected and observed fragment sizes with a particular digest and probe, based on the known restriction enzyme sites

30

of the cry1Ac and cry1F PTU, are given in Table 7. Two types of fragments were identified from these digests and hybridizations: internal fragments where known enzyme sites flank the probe region and are completely contained within the insertion region of the cry1Ac and cry1F PTU, and border fragments where a known enzyme site is located at one end of the probe region and a second site is expected in the soybean genome. Border fragment sizes vary by event because, in most cases, DNA fragment integration sites are unique for each event. The border fragments provide a means to locate a restriction enzyme site relative to the integrated DNA and to evaluate the number of DNA insertions. Southern blot analyses completed on multiple generations of soybean containing soybean event pDAB9582.814.19.1 produced data which suggested that a low copy, intact cry1Ac and cry1F PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.814.19.1.

TABLE 7

Predicted and observed hybridizing fragments in Southern blot analysis.				
DNA Probe	Restriction Enzymes	Samples	Expected Fragment Sizes (bp) <sup>1</sup>	Observed Fragment Size (bp) <sup>2</sup>
Cry1Ac	AseI	pDAB9582	13476	>14000
		Maverick	none	none
		Soybean Event	>7286	~7400
		pDAB9582.814.19.1		
	Nsi I	pDAB9582	15326	>15000
		Maverick	none	none
		Soybean Event	>9479	>10000
		pDAB9582.814.19.1		
	Not I + ApaLI	pDAB9582	4550	~4500
		Maverick	none	none
		Soybean Event	4550	~4500
		pDAB9582.814.19.1		
Cry1F	NdeI	pDAB9582	8071	~8000
		Maverick	none	none
		Soybean Event	5569	~7500
		pDAB9582.814.19.1		
	Nsi I	pDAB9582	11044	11000
		Maverick	none	none
		Soybean Event	>9479	>10000
		pDAB9582.814.19.1		
	Hind III	pDAB9582	7732	~7700
		Maverick	none	none
		Soybean Event	7732	~7700
		pDAB9582.814.19.1		
SpecR	NsiI	pDAB9582	15320	~15000
		Maverick	none	none
		Soybean Event	none	none
		pDAB9582.814.19.1		
	trfA	pDAB9582	15320	~15000
		Maverick	none	none
		Soybean Event	none	none
		pDAB9582.814.19.1		
	oriREP	pDAB9582	5239	~5000
		Maverick	none	none
		Soybean Event	none	none
		pDAB9582.814.19.1		

<sup>1</sup>Expected fragment sizes are based on the plasmid map of pDAB9582.

<sup>2</sup>Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-LABELED DNA MOLECULAR WEIGHT MARKER II and MARK VII fragments.

The restriction enzymes AseI and NsiI bind and cleave unique restriction sites in plasmid pDAB9582. Subsequently, these enzymes were selected to characterize the cry1Ac gene insert in soybean event pDAB9582.814.19.1. Border fragments of >7286 bp or >9479 bp were predicted to hybridize with the probe following AseI and NsiI digests, respectively (Table 7). Single cry1Ac hybridization bands of about 7400 and >10000 bp were observed when AseI and NsiI digests were used, respectively. The hybridization of the probe to

## US 8,680,363 B2

## 31

bands of this size suggests the presence of a single site of insertion for the cry1Ac gene in the soybean genome of soybean event pDAB9582.814.19.1. Restriction enzymes NotI and ApaLI were selected to perform a double digestion and to release a fragment which contains the cry1Ac plant transcription unit (PTU; promoter/gene/terminator) (Table 7). The predicted 4550 bp fragments were observed with the probe following NotI and ApaLI double digestion. Results obtained with the enzyme digestion of the pDAB9582.814.19.1 samples followed by probe hybridization indicated that an intact cry1Ac PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.814.19.1.

The restriction enzymes NdeI and NsiI bind and cleave restriction sites in plasmid pDAB9582. Subsequently, these enzymes were selected to characterize the cry1F gene insert in soybean event pDAB9582.814.19.1. Border fragments of >5569 bp and >9479 were predicted to hybridize with the probe following the NdeI and NsiI digests, respectively (Table 7). Single cry1F hybridization bands of ~7500 bp and >10000 bp were observed when NdeI and NsiI were used, respectively. The hybridization of the probe to bands of this size suggests the presence of a single site of insertion for the cry1F gene in the soybean genome of soybean event pDAB9582.814.19.1. Restriction enzyme, HindIII, was selected to release a fragment which contains the cry1F plant transcription unit (PTU; promoter/gene/terminator) (Table 7). The predicted 7732 bp fragment was observed with the probe following the HindIII digestions. Results obtained with the enzyme digestion of the pDAB9582.814.19.1 samples followed by probe hybridization indicated that an intact cry1F PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.814.19.1.

## Example 4.6

## Absence of Backbone Sequences

Southern blot analysis was also conducted to verify the absence of the spectinomycin resistance gene (specR), On Rep element and replication initiation protein trfA (trf A element) in soybean event pDAB9582.814.19.1. No specific hybridization to spectinomycin resistance, On Rep element or trf A element is expected when appropriate positive (pDAB9582 added to Maverick genomic DNA) and negative (Maverick genomic DNA) controls are included for Southern analysis. Following the NsiI digestion and hybridization with the specR specific probe, one expected size band of 15320 bp was observed in the positive control sample (pDAB9582 added to Maverick genomic DNA). The specR probe did not

## 32

hybridize to samples of the negative control and soybean event pDAB9582.814.19.1. Similarly, one expected size band of 15320 bp was detected in the positive control sample (pDAB9582 plus maverick) but absent from the samples of the negative control and soybean event pDAB9582.814.19.1 after NsiI digestion and hybridization with trfA probe. Another expected size band of 5329 bp was detected in the positive control sample (pDAB9582 added to Maverick genomic DNA) but absent from the samples of the negative control and soybean event pDAB9582.814.19.1 after NdeI digestion and hybridization with OriRep specific probe. These data indicate the absence of spectinomycin resistance gene, Ori Rep element and replication initiation protein trfA in soybean event pDAB9582.814.19.1.

## Example 5

## Agronomic and Yield Field Trial and Herbicide Tolerance

To test the agronomic characteristics and efficacy of soybean event pDAB9582.814.19.1 the event was planted in an efficacy trial at Santa Isabel, Puerto Rico in October 2010 and February 2011. The cultivar Maverick, which was originally transformed to produce event pDAB9582.814.19.1, was planted in each nursery and included as a control in the experiments. Seed for the T3 nursery was derived from single plant selections at the T2 stage and seed for the T4 nursery was derived from single plant selections at the T3 stage. Four lineages of the event were tested each generation. Each lineage was planted in a plot which was 4 rows wide and 7.5 feet long. The spacing between rows was 30 inches. Plots were grown under lights for approximately 2.5 weeks to compensate for the short day length in Puerto Rico. Each nursery was sprayed with glufosinate at a rate of 411 g ae/ha. One plot of the control plants, Maverick, was sprayed with the same rate of glufosinate and a second plot was non-sprayed and used as control comparison for the event.

Data was collected on emergence, general appearance, vigor, height, lodging, and maturity. Herbicide tolerance was assessed by visually looking for chlorosis, leaf necrosis and plant death (Table 8).

For comparisons of soybean event pDAB9582.814.19.1 with Maverick, only data from the unsprayed block of Maverick were used. For comparison of the sprayed and non-sprayed treatments, data from the soybean event pDAB9582.814.19.1 block sprayed with a given treatment were compared with data from the Maverick control non-sprayed block. Soybean event pDAB9582.814.19.1 showed tolerance to the glufosinate herbicide application. In contrast, none of the Maverick plants were tolerant to the herbicide treatments.

TABLE 8

Comparison of soybean event pDAB9582.814.19.1 to Maverick. Values are averages from T<sub>3</sub> and T<sub>4</sub> nurseries. Each nursery of soybean event pDAB9582.814.19.1 was sprayed with glufosinate at the V3 stage at a rate of 411 g ae/ha.

Event	Emergence (%)	Appearance	Vigor	Height (cm)	Lodging (%)	Maturity (day)
		(1 = poor to 9 = good)	(1 = poor to 9 = good)			
pDAB9582.814.19.1	90	8	8	69	1	91
Maverick	82	8	8	64	1	91

US 8,680,363 B2

33

Example 6

Characterization of Insecticidal Activity for Soybean  
Event 9582.814.19.1

Field and greenhouse evaluations were conducted to characterize the activity of Cry1Ac and Cry1F in soybean event pDAB9582.814.19.1 against lab reared soybean pests including *Anticarsia gemmatilis* (velvetbean caterpillar), *Pseudoplusia includens* (soybean looper) and *Spodoptera frugiperda* (fall armyworm). Soybean event pDAB9582.814.19.1 was compared against non-transformed soybean variety Maverick, to determine the level of plant protection provided by the Cry1F and Cry1Ac proteins.

Greenhouse trials were conducted on approximately four week old plants. Fifteen plants were used to evaluate the soybean event pDAB9582.814.19.1 and the Maverick control. For each insect species tested (*Anticarsia gemmatilis*, *Pseudoplusia includes*, and *Spodoptera frugiperda*) 3 leaf punches were made from each plant for a total of 45 leaf discs/plant/insect species. The 1.4 cm diameter (or 1.54 cm<sup>2</sup>) leaf punches were placed in a test arena on top of 2% water agar, infested with one neonate larvae and sealed with a perforated plastic lid. Mortality and leaf consumption were rated 4 days after infestation. Larvae that were not responsive to gentle probing were considered dead. Leaf damage was assessed by visually scoring the percentage of leaf punch consumed by the insect.

Field evaluations were conducted by collecting leaf samples from seed increase nursery plots in Santa Isabel, Puerto Rico and sending these leaves to Indianapolis, Ind. for testing. The nursery plot for soybean event pDAB9582.814.19.1 was planted in February 2011 and consisted of approximately 180 plants arranged in four rows. Each row was 2.3 m long and spaced 76.2 cm apart; individual plants were spaced 5.1 cm apart within each row. In March 2011, one fully-expanded, mainstem trifoliate leaf, located approximately four nodes below the meristem, was excised from 10 soybean event pDAB9582.814.19.1 plants and 10 'Maverick' plants. The leaves were placed in labeled plastic bags, (one per bag) and sealed. The bagged leaves were packed and transferred to the laboratory. In the laboratory, one or two 3.33 cm (1.31 in) diameter leaf discs were punched from each trifoliate leaf to provide a total of 16 leaf discs. Each leaf disc was placed in a test arena on top of 2% agar, infested with one neonate *S. frugiperda* larva, and sealed with a perforated plastic lid. The leaf discs were held in a controlled environment chamber for 7 days, at which time mortality and leaf consumption were rated. Larvae not responsive to gentle probing were considered dead. Leaf damage was assessed by visually scoring the percentage of leaf punch consumed by the insect.

The results obtained from these replicated experiments indicated the soybean event pDAB9582.814.19.1 sustained significantly lower damage than the Maverick control plants for all insects tested. Thus, the soybean event pDAB9582.814.19.1 has insecticidal activity over this broad host range.

34

Example 7

## Sequence of Soybean Event pDAB9582.814.19.1

5 SEQ ID NO:14 provides the sequence of soybean event pDAB9582.814.19.1. This sequence contains the 5' genomic flanking sequence, the T-strand insert of pDAB9582 and 3' genomic flanking sequences. With respect to SEQ ID NO:14, residues 1-1400 are 5' genomic flanking sequence, residues 1401-1536 are residues of a rearrangement from the pDAB9582 plasmid and 1537-13896 are residues of the pDAB9582 T-strand insert, and residues 13897-15294 are 3' flanking sequence. The junction sequence or transition with respect to the 5' end of the insert thus occurs at residues 1400-1401 of SEQ ID NO:14. The junction sequence or transition with respect to the 3' end of the insert thus occurs at residues 13896-13897 of SEQ ID NO:14.

It should be noted that progeny from soybean event pDAB9582.814.19.1 may have sequences which slightly deviate from SEQ ID NO:14. During the introgression and breeding process of introducing soybean event pDAB9582.814.19.1 into the genome of plant cells, it is not uncommon for some deletions or other alterations of the insert to occur. Moreover, errors in PCR amplification can occur which might result in minor sequencing errors. For example, flanking sequences listed herein were determined by generating amplicons from soybean genomic DNAs, and then cloning and sequencing the amplicons. It is not unusual to find slight differences and minor discrepancies in sequences generated and determined in this manner, given the many rounds of amplification that are necessary to generate enough amplicon for sequencing from genomic DNAs. One skilled in the art should recognize and be put on notice that any adjustments needed due to these types of common sequencing errors or discrepancies are within the scope of the subject invention. Thus, the relevant segment of the plasmid sequence provided herein might comprise some minor variations. Thus, a plant comprising a polynucleotide having some range of identity with the subject insert sequence is within the scope of the subject invention. Identity to the sequence of SEQ ID NO:14 can be a polynucleotide sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with a sequence exemplified or described herein. Thus, some differences between SEQ ID NO:14 and soybean event pDAB9582.814.19.1 progeny plants may be identified and are within scope of the present invention.

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

## SEQUENCE LISTING

&lt;160&gt; NUMBER OF SEQ ID NOS: 14

&lt;210&gt; SEQ ID NO 1

&lt;211&gt; LENGTH: 1836

US 8,680,363 B2

35

36

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&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Glycine max

&lt;400&gt; SEQUENCE: 1

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&lt;213&gt; ORGANISM: Glycine max

&lt;400&gt; SEQUENCE: 2

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US 8,680,363 B2

37

38

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&lt;211&gt; LENGTH: 12381

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Plasmid sequence of pDAB9582

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US 8,680,363 B2

39

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## US 8,680,363 B2

41

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## US 8,680,363 B2

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US 8,680,363 B2

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US 8,680,363 B2

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US 8,680,363 B2

49

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US 8,680,363 B2

51

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US 8,680,363 B2

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## US 8,680,363 B2

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US 8,680,363 B2

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## US 8,680,363 B2

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US 8,680,363 B2

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US 8,680,363 B2

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The invention claimed is:

1. A method of controlling insects, said method comprising exposing insects to insect resistant soybean plants, said soybean plants comprising SEQ ID NO: 14, wherein said insects are selected from the group consisting of: *Pseudoplusia*

*includens* (soybean looper), *Anticarsia gemmatilis* (velvet bean caterpillar) and *Spodoptera frugiperda* (fall armyworm).

2. The method of claim 1 wherein said insects are *Pseudoplusia includens* (soybean looper).

US 8,680,363 B2

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3. The method of claim 1 wherein said insects are *Anticarsia gemmatilis* (velvet bean caterpillar).

4. The method of claim 1 wherein said insects are *Spodoptera frugiperda* (fall armyworm).

5. A method of controlling weeds in a soybean crop, said method comprising applying glufosinate herbicide to the soybean crop, said soybean crop comprising soybean plants comprising SEQ ID NO:14.

6. A method of breeding a soybean plant, said method comprising: crossing a first plant with a second soybean plant to produce a third soybean plant, said first plant comprising DNA comprising SEQ ID NO:14; and assaying said third soybean plant for the presence of SEQ ID NO:14.

7. A soybean plant, wherein representative seed of said soybean plant has been deposited with the American Type Culture Collection under Accession No. PTA-12006.

8. A seed or a part of the plant of claim 7, wherein said seed or part comprises SEQ ID NO:14.

9. A soybean plant, or part thereof, comprising the DNA sequence of SEQ ID NO: 14.

\* \* \* \* \*

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Fig. 1

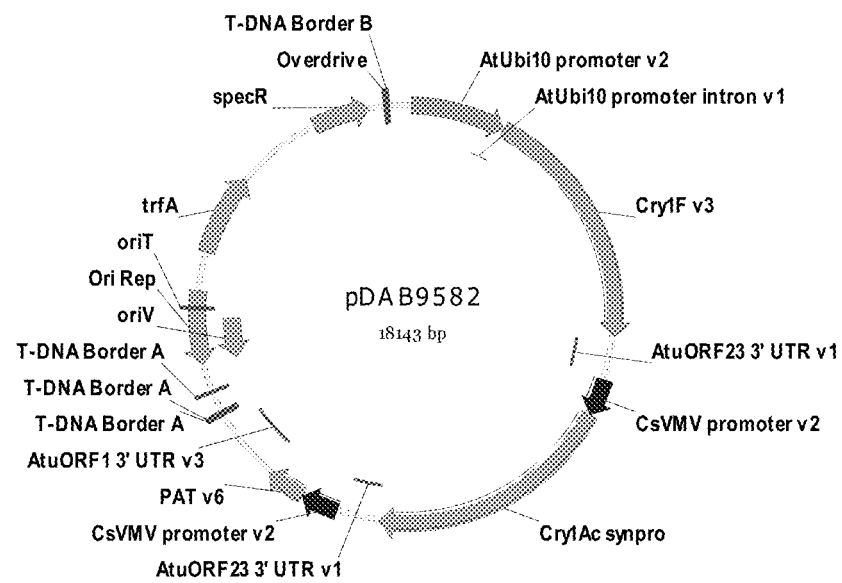


Fig. 2

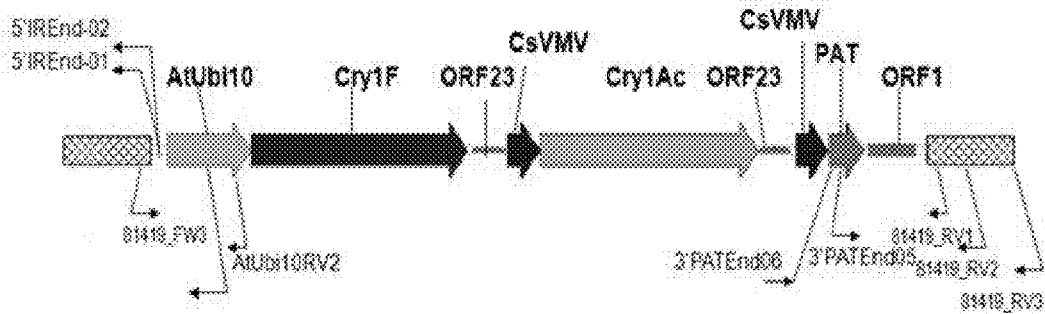
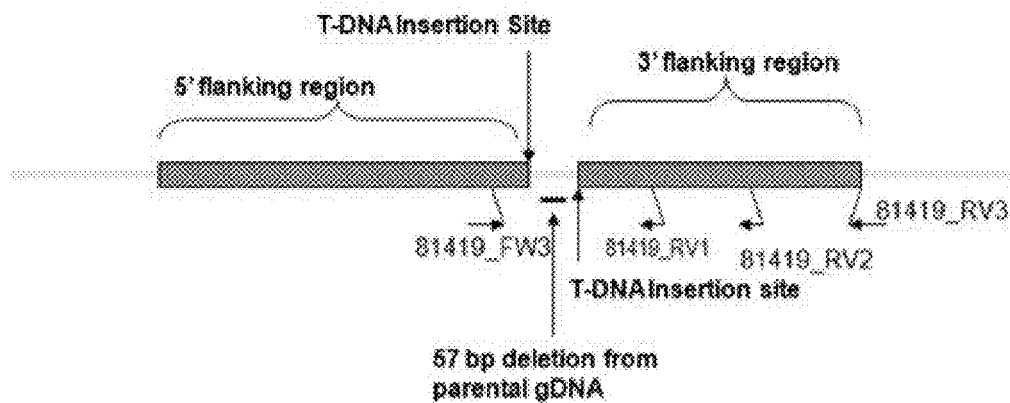


Fig. 3



UNITED STATES PATENT AND TRADEMARK OFFICE  
**CERTIFICATE OF CORRECTION**

PATENT NO. : 8,680,363 B2  
APPLICATION NO. : 13/559177  
DATED : March 25, 2014  
INVENTOR(S) : Nathan Bard et al.

Page 1 of 1

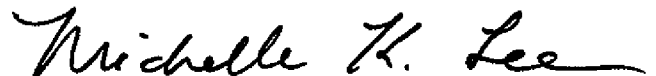
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

In The Claims

Column 65, Line 2 should read as follows:

sia gemmatalis (velvet bean caterpillar).

Signed and Sealed this  
Sixth Day of January, 2015

A handwritten signature in black ink, reading "Michelle K. Lee". The signature is fluid and cursive, with the first letters of the first and last names being capitalized and prominent.

Michelle K. Lee  
*Deputy Director of the United States Patent and Trademark Office*

# **Exhibit F**



(12) **United States Patent**  
**Bard et al.**

(10) **Patent No.:** **US 9,695,441 B2**

(45) **Date of Patent:** **\*Jul. 4, 2017**

(54) **INSECT RESISTANT AND HERBICIDE  
TOLERANT SOYBEAN EVENT 9582.814.19.1**

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(71) Applicant: **DOW AGROSCIENCES LLC**,  
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IN (US)

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(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 504 days.

This patent is subject to a terminal dis-  
claimer.

(21) Appl. No.: **14/223,249**

(22) Filed: **Mar. 24, 2014**

(65) **Prior Publication Data**

US 2014/0201873 A1 Jul. 17, 2014

**Related U.S. Application Data**

(63) Continuation of application No. 13/559,177, filed on  
Jul. 26, 2012, now Pat. No. 8,680,363.

(60) Provisional application No. 61/511,664, filed on Jul.  
26, 2011, provisional application No. 61/521,798,  
filed on Aug. 10, 2011.

(51) **Int. Cl.**

<b>C12N 15/82</b>	(2006.01)
<b>C07H 21/04</b>	(2006.01)
<b>A01H 5/00</b>	(2006.01)
<b>A01H 5/10</b>	(2006.01)
<b>C12Q 1/68</b>	(2006.01)
<b>A01N 37/38</b>	(2006.01)
<b>A01N 37/44</b>	(2006.01)
<b>A01N 65/20</b>	(2009.01)

(52) **U.S. Cl.**

CPC ..... **C12N 15/8286** (2013.01); **A01N 37/38**  
(2013.01); **A01N 37/44** (2013.01); **A01N**  
**65/20** (2013.01); **C12N 15/8209** (2013.01);  
**C12N 15/8274** (2013.01); **C12N 15/8277**  
(2013.01); **C12Q 1/6895** (2013.01); **C12Q**  
**2600/13** (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(Continued)

*Primary Examiner* — Mykola V Kovalenko

(74) *Attorney, Agent, or Firm* — Ronald S. Maciak;  
Barnes & Thornburg LLP

(57) **ABSTRACT**

Soybean event 9582.814.19.1 comprising genes encoding  
Cry1F, Cry1Ac (synpro), and PAT, affording insect resis-  
tance and herbicide tolerance to soybean crops containing  
the event, and enabling methods for crop protection and  
protection of stored products.

**4 Claims, 2 Drawing Sheets**

## US 9,695,441 B2

Page 2

(56)

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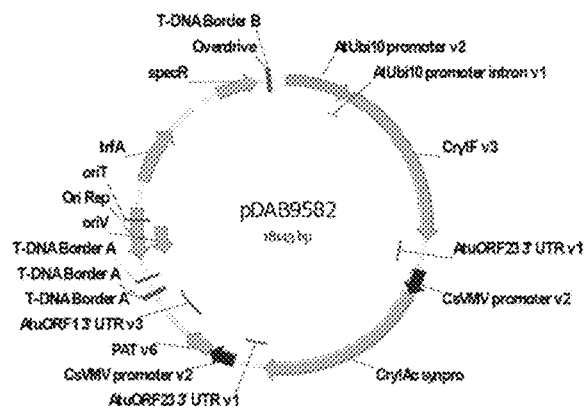
U.S. Patent

Jul. 4, 2017

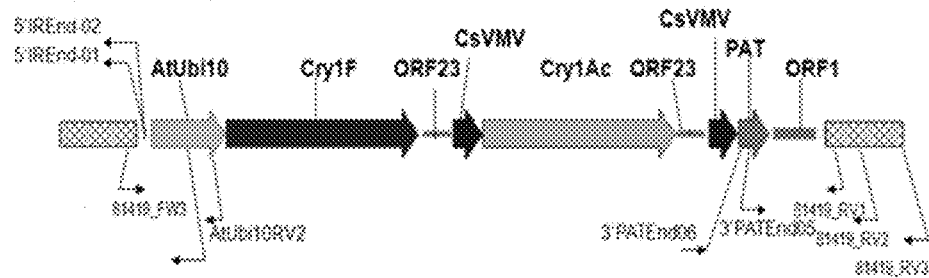
Sheet 1 of 2

US 9,695,441 B2

**Figure 1.** is a plasmid Map of pDAB9582 containing the *cry1F* v3, *cry1Ac* and *pat* v6 expression cassette.



**Figure 2.** The diagram depicts the primer locations for confirming the 5' and 3' border sequence of the soybean event pDAB9582.814.19.1.



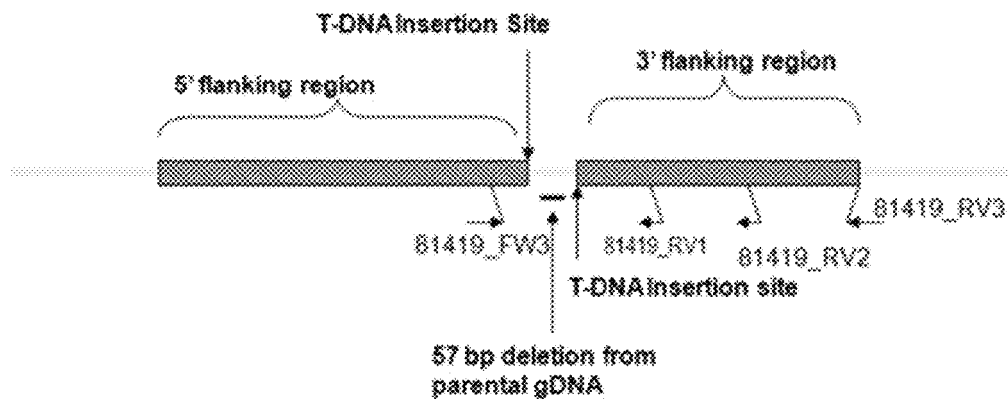
U.S. Patent

Jul. 4, 2017

Sheet 2 of 2

US 9,695,441 B2

**Figure 3.** The diagram depicts the genomic sequence arrangement in soybean event pDAB9582.814.19.1



US 9,695,441 B2

1

**INSECT RESISTANT AND HERBICIDE  
TOLERANT SOYBEAN EVENT 9582.814.19.1****CROSS-REFERENCE TO RELATED  
APPLICATIONS**

This application is a continuation of U.S. application Ser. No. 13/559,177, filed Jul. 26, 2012, which claims benefit of Provisional Application No. 61/511,664, filed Jul. 26, 2011, and Provisional Application No. 61/521,798, filed Aug. 10, 2011, all of which are herein incorporated by reference in their entireties.

**BACKGROUND OF INVENTION**

The genes encoding Cry1F and Cry1Ac synpro (Cry1Ac) are capable of imparting insect resistance, e.g. resistance to lepidopteran insects, to transgenic plants; and the gene encoding PAT (phosphinothricin acetyltransferase) is capable of imparting tolerance to the herbicide phosphinothricin (glufosinate) to transgenic plants. PAT has been successfully expressed in soybean for use both as a selectable marker in producing insect resistant transgenic crops, and to impart commercial levels of tolerance to the herbicide glufosinate in transgenic crops.

The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site (Weising et al., *Ann. Rev. Genet.* 22:421-477, 1988). At the same time the presence of the transgene at different locations in the genome will influence the overall phenotype of the plant in different ways. For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of an introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant tissues, that may not correspond to the patterns expected from transcriptional regulatory elements present in the introduced gene construct. For this reason, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual outcrossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure reliable gene expression in a number of varieties that are well adapted to local growing conditions.

It is desirable to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene or group of transgenes of interest. In addition, a method for detecting a particular event would be helpful for complying with regulations requiring the pre-market approval and labeling of foods derived from recombinant crop plants, for example, or for use in environmental monitoring, monitoring traits in crops in the field, or monitoring products derived from a crop harvest, as well as for use in ensuring compliance of parties subject to regulatory or contractual terms.

2

It is possible to detect the presence of a transgenic event by any nucleic acid detection method known in the art including, but not limited to, the polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc., because for many DNA constructs, the coding region is interchangeable. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known. For example, an event-specific PCR assay is described in United States Patent Application 2006/0070139 for maize event DAS-59122-7. It would be desirable to have a simple and discriminative method for the identification of soybean event 9582.814.19.1.

**BRIEF SUMMARY OF THE INVENTION**

The present invention relates to a new insect resistant and herbicide tolerant transgenic soybean transformation event, designated soybean event 9582.814.19.1, comprising cry1F, cry1Ac and pat, as described herein, inserted into a specific site within the genome of a soybean cell. Representative soybean seed has been deposited with American Type Culture Collection (ATCC) with the Accession No. as described herein. The DNA of soybean plants containing this event includes the junction/flanking sequences described herein that characterize the location of the inserted DNA within the soybean genome. SEQ ID NO:1 and SEQ ID NO:2 are diagnostic for soybean event 9582.814.19.1. More particularly, sequences surrounding the junctions at bp 1400/1401, and bp 1536/1537 of SEQ ID NO:1, and bp 152/153 of SEQ ID NO:2 are diagnostic for soybean event 9582.814.19.1. Described herein are examples of sequences comprising these junctions that are characteristic of DNA of soybeans containing soybean event 9582.814.19.1.

In one embodiment, the invention provides a soybean plant, or part thereof, that is resistant to *Pseudoplusia includens* (soybean looper) and that has a genome comprising one or more sequences selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof. In another embodiment, the invention provides seed of such plants.

In another embodiment, the invention provides a method of controlling insects that comprises exposing insects to insect resistant soybean plants, wherein the soybean plants have a genome that contains one or more sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof; which are characteristic of the presence of soybean event 9582.814.19.1, to thereby control the insects. Presence of the cry1F v3 (cry1F) and cry1Ac synpro (cry1Ac) genes in soybean event 9582.814.19.1 imparts resistance to, for example, *Pseudoplusia includens* (soybean looper), *Anticarsia gemmatilis* (velvetbean caterpillar), *Epinotia aporema*, *Omoidea indicatus*, *Rachiplusia nu*, *Spodoptera frugiperda*, *Spodoptera cosmoides*, *Spodoptera eridania*, *Heliothis virescens*, *Helioverpa zea*, *Spilosoma virginica* and *Elasmopalpus lignosellus*.

US 9,695,441 B2

3

In another embodiment, the invention provides a method of controlling weeds in a soybean crop that comprises applying glufosinate herbicide to the soybean crop, said soybean crop comprising soybean plants that have a genome containing one or more sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof, which are diagnostic for the presence of soybean event 9582.814.19.1. Presence of the pat v6 (pat) gene in soybean event 9582.814.19.1 imparts tolerance to glufosinate herbicide.

In another embodiment, the invention provides a method of detecting soybean event 9582.814.19.1 in a sample comprising soybean DNA, said method comprising:

- (a) contacting said sample with a first primer at least 10 bp in length that selectively binds to a flanking sequence within bp 1-1400 of SEQ ID NO:1 or the complement thereof, and a second primer at least 10 bp in length that selectively binds to an insert sequence within bp 1401-1836 of SEQ ID NO:1 or the complement thereof; and assaying for an amplicon generated between said primers; or
- (b) contacting said sample with a first primer at least 10 bp in length that selectively binds to an insert sequence within bp 1-152 of SEQ ID NO:2 or the complement thereof, and a second primer at least 10 bp in length that selectively binds to flanking sequence within bp 153-1550 of SEQ ID NO:2 or the complement thereof; and
- (c) assaying for an amplicon generated between said primers.

In another embodiment, the invention provides a method of detecting soybean event 9582.814.19.1 comprising:

- (a) contacting said sample with a first primer that selectively binds to a flanking sequence selected from the group consisting of bp 1-1400 of SEQ ID NO:1 and bp 153-1550 of SEQ ID NO:2, and complements thereof; and a second primer that selectively binds to SEQ ID NO:3, or the complement thereof;
- (b) subjecting said sample to polymerase chain reaction; and
- (c) assaying for an amplicon generated between said primers.

In another embodiment the invention provides a method of breeding a soybean plant comprising: crossing a first plant with a second soybean plant to produce a third soybean plant, said first plant comprising DNA comprising one or more sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof; and assaying said third soybean plant for presence of DNA comprising one or more sequences selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof.

In another embodiment the invention provides an isolated DNA molecule that is diagnostic for soybean event 9582.814.19.1. Such molecules include, in addition to SEQ ID NOS: 1 and 2, molecules at least 25 bp in length comprising bp 1400-1401 of SEQ ID NO:1 and at least 10 bp of SEQ ID NO:1 in each direction from the bp 1400/1401 junction; amplicons at least 25 bp in length comprising 152-153 of SEQ ID NO:2 and at least 10 bp of SEQ ID NO:2

4

in each direction from the bp 152/153 junction. Examples are bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof.

In another embodiment the invention provides a method of controlling pests in soybean grain, seed, or seed meal which comprises including soybean event 9582.814.19.1 in said grain, seed, or seed meal as demonstrated by said grain, seed, or seed meal comprising DNA comprising one or more sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1; bp 1350-1450 of SEQ ID NO:1; bp 1300-1500 of SEQ ID NO:1; bp 1200-1600 of SEQ ID NO:1; bp 137-168 of SEQ ID NO:2; bp 103-203 of SEQ ID NO:2; and bp 3-303 of SEQ ID NO:2, and complements thereof.

The invention also includes soybean plant cells and plant parts including, but are not limited to pollen, ovule, flowers, shoots, roots, and leaves, and nuclei of vegetative cells, pollen cells, seed and seed meal, and egg cells, that contain soybean event 9582.814.19.1.

In some embodiments, soybean event 9582.814.19.1 can be combined with other traits, including, for example, other herbicide tolerance gene(s) and/or insect-inhibitory proteins and transcription regulatory sequences (i.e. RNA interference, dsRNA, transcription factors, etc). The additional traits may be stacked into the plant genome via plant breeding, re-transformation of the transgenic plant containing soybean event 9582.814.19.1, or addition of new traits through targeted integration via homologous recombination.

Other embodiments include the excision of polynucleotide sequences which comprise soybean event 9582.814.19.1, including for example, the pal gene expression cassette. Upon excision of a polynucleotide sequence, the modified event may be re-targeted at a specific chromosomal site wherein additional polynucleotide sequences are stacked with soybean event 9582.814.19.1.

In one embodiment, the present invention encompasses a soybean chromosomal target site located on chromosome 02 between the flanking sequences set forth in SEQ ID NOS:1 and 2.

In one embodiment, the present invention encompasses a method of making a transgenic soybean plant comprising inserting a heterologous nucleic acid at a position on chromosome 02 between the genomic sequences set forth in SEQ ID NOS:1 and 2, i.e. between bp 1-1400 of SEQ ID NO:1 and bp 153-1550 of SEQ ID NO:2.

Additionally, the subject invention provides assays for detecting the presence of the subject event in a sample (of soybeans, for example). The assays can be based on the DNA sequence of the recombinant construct, inserted into the soybean genome, and on the genomic sequences flanking the insertion site. Kits and conditions useful in conducting the assays are also provided.

The subject invention relates in part to the cloning and analysis of the DNA sequences of the border regions resulting from insertion of T-DNA from pDAB9582 in transgenic soybean lines. These sequences are unique. Based on the insert and junction sequences, event-specific primers can be and were generated. PCR analysis demonstrated that these events can be identified by analysis of the PCR amplicons generated with these event-specific primer sets. Thus, these and other related procedures can be used to uniquely identify soybean lines comprising the event of the subject invention.

#### SEED DEPOSIT

As part of this disclosure at least 2500 seeds of a soybean line comprising soybean event 9582.814.19.1 were depos-



US 9,695,441 B2

5

ited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, Va., 20110. The deposit, ATCC Patent Deposit Designation, PTA-12006, was received by the ATCC on Jul. 21, 2011. This deposit was made and will be maintained in accordance with and under the terms of the Budapest Treaty with respect to seed deposits for the purposes of patent procedure. This deposit was made and will be maintained in accordance with and under the terms of the Budapest Treaty with respect to seed deposits for the purposes of patent procedure.

#### BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the 5' DNA flanking border sequence for soybean event 9582.814.19.1. Nucleotides 1-1400 are genomic sequence. Nucleotides 1401-1535 are a rearranged sequence from pDAB9582. Nucleotides 1536-1836 are insert sequence.

SEQ ID NO:2 is the 3' DNA flanking border sequence for soybean event 9582.814.19.1. Nucleotides 1-152 are insert sequence. Nucleotides 153-1550 are genomic sequence.

SEQ ID NO:3 is the DNA sequence of pDAB9582, which is annotated below in Table 1.

SEQ ID NO:4 is oligonucleotide primer 81419\_FW3 for confirmation of 5' border genomic DNA.

SEQ ID NO:5 is oligonucleotide primer 81419\_RV1 for confirmation of 3' border genomic DNA.

SEQ ID NO:6 is oligonucleotide primer 81419\_RV2 for confirmation of 3' border genomic DNA.

SEQ ID NO:7 is oligonucleotide primer 81419\_RV3 for confirmation of 3' border genomic DNA.

SEQ ID NO:8 is oligonucleotide primer 5'IREnd-01 for confirmation of 5' border genomic DNA.

SEQ ID NO:9 is oligonucleotide primer 5'IREnd-02 for confirmation of 5' border genomic DNA.

SEQ ID NO:10 is oligonucleotide primer AtUbi10RV1 for confirmation of 5' border genomic DNA.

SEQ ID NO:11 is oligonucleotide primer AtUbi10RV2 for confirmation of 5' border genomic DNA.

SEQ ID NO:12 is oligonucleotide primer 3'PATEnd05 for confirmation of 3' border genomic DNA.

SEQ ID NO:13 is oligonucleotide primer 3'PATEnd06 for confirmation of 3' border genomic DNA.

SEQ ID NO:14 is the confirmed sequence of soybean event 9582.814.19.1. Including the 5' genomic flanking sequence, pDAB9582 T-strand insert, and 3' genomic flanking sequence.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a plasmid Map of pDAB9582 containing the cry1F, cry1Ac and pat expression cassettes.

FIG. 2 depicts the primer locations for confirming the 5' and 3' border sequence of the soybean event pDAB9582.814.19.1.

FIG. 3 depicts the genomic sequence arrangement in soybean event pDAB9582.814.19.1

#### DETAILED DESCRIPTION OF THE INVENTION

Both ends of the soybean event 9582.814.19.1 insertion have been sequenced and characterized. Event specific assays were developed. It has also been mapped onto the soybean genome (soybean chromosome 02). The event can be introgressed into further elite lines.

6

As alluded to above in the Background section, the introduction and integration of a transgene into a plant genome involves some random events (hence the name "event" for a given insertion that is expressed). That is, with many transformation techniques such as *Agrobacterium* transformation, the biolistic transformation (i.e. gene gun), and silicon carbide mediated transformation (i.e. WHISKERS), it is unpredictable where in the genome a transgene will become inserted. Thus, identifying the flanking plant genomic DNA on both sides of the insert can be important for identifying a plant that has a given insertion event. For example, PCR primers can be designed that generate a PCR amplicon across the junction region of the insert and the host genome. This PCR amplicon can be used to identify a unique or distinct type of insertion event.

Definitions and examples are provided herein to help describe the present invention and to guide those of ordinary skill in the art to practice the invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. The nomenclature for DNA bases as set forth at 37 CFR §1.822 is used.

As used herein, the term "progeny" denotes the offspring of any generation of a parent plant which comprises soybean event 9582.814.19.1.

A transgenic "event" is produced by transformation of plant cells with heterologous DNA, i.e., a nucleic acid construct that includes the transgenes of interest, regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. The term "event" refers to the original transformant and progeny of the transformant that include the heterologous DNA. The term "event" also refers to progeny produced by a sexual outcross between the transformant and another variety that includes the genomic/transgene DNA. Even after repeated back-crossing to a recurrent parent, the inserted transgene DNA and flanking genomic DNA (genomic/transgene DNA) from the transformed parent is present in the progeny of the cross at the same chromosomal location. The term "event" also refers to DNA from the original transformant and progeny thereof comprising the inserted DNA and flanking genomic sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

A "junction sequence" or "border sequence" spans the point at which DNA inserted into the genome is linked to DNA from the soybean native genome flanking the insertion point, the identification or detection of one or the other junction sequences in a plant's genetic material being sufficient to be diagnostic for the event. Included are the DNA sequences that span the insertions in herein-described soybean events and similar lengths of flanking DNA. Specific examples of such diagnostic sequences are provided herein; however, other sequences that overlap the junctions of the insertions, or the junctions of the insertions and the genomic sequence, are also diagnostic and could be used according to the subject invention.

The subject invention relates in part to event identification using such flanking, junction, and insert sequences. Related PCR primers and amplicons are included in the invention. According to the subject invention, PCR analysis methods

US 9,695,441 B2

7

using amplicons that span across inserted DNA and its borders can be used to detect or identify commercialized transgenic soybean varieties or lines derived from the subject proprietary transgenic soybean lines.

The flanking/junction sequences are diagnostic for soybean event 9582.814.19.1. Based on these sequences, event-specific primers were generated. PCR analysis demonstrated that these soybean lines can be identified in different soybean genotypes by analysis of the PCR amplicons generated with these event-specific primer sets. Thus, these and other related procedures can be used to uniquely identify these soybean lines. The sequences identified herein are unique.

Detection techniques of the subject invention are especially useful in conjunction with plant breeding, to determine which progeny plants comprise a given event, after a parent plant comprising an event of interest is crossed with another plant line in an effort to impart one or more additional traits of interest in the progeny. These PCR analysis methods benefit soybean breeding programs as well as quality control, especially for commercialized transgenic soybean seeds. PCR detection kits for these transgenic soybean lines can also now be made and used. This can also benefit product registration and product stewardship.

Furthermore, flanking soybean/genomic sequences can be used to specifically identify the genomic location of each insert. This information can be used to make molecular marker systems specific to each event. These can be used for accelerated breeding strategies and to establish linkage data.

Still further, the flanking sequence information can be used to study and characterize transgene integration processes, genomic integration site characteristics, event sorting, stability of transgenes and their flanking sequences, and gene expression (especially related to gene silencing, transgene methylation patterns, position effects, and potential expression-related elements such as MARS [matrix attachment regions], and the like).

In light of all the subject disclosure, it should be clear that the subject invention includes seeds available under the ATCC Deposit No. as described herein. The subject invention also includes a herbicide-tolerant soybean plant grown from a seed deposited with the ATCC Deposit No. as described herein. The subject invention further includes parts of said plant, such as leaves, tissue samples, seeds produced by said plant, pollen, and the like (wherein they comprise cry1F, cry1Ac, pat, and SEQ ID NOS: 1 and 2).

Still further, the subject invention includes descendant and/or progeny plants of plants grown from the deposited seed, preferably a herbicide-resistant soybean plant wherein said plant has a genome comprising a detectable wild-type junction sequence as described herein. As used herein, the term "soybean" means *Glycine max* and includes all varieties thereof that can be bred with a soybean plant.

This invention further includes processes of making crosses using a plant of the subject invention as at least one parent. For example, the subject invention includes an F<sub>1</sub> hybrid plant having as one or both parents any of the plants exemplified herein. Also within the subject invention is seed produced by such F<sub>1</sub> hybrids of the subject invention. This invention includes a method for producing an F<sub>1</sub> hybrid seed by crossing an exemplified plant with a different (e.g., in-bred parent) plant and harvesting the resultant hybrid seed. The subject invention includes an exemplified plant that is either a female parent or a male parent. Characteristics of the resulting plants may be improved by careful consideration of the parent plants.

An insect resistant/glufosinate-tolerant soybean plant of the subject invention can be bred by first sexually crossing

8

a first parental soybean plant consisting of a soybean plant grown from seed of any one of the lines referred to herein, and a second parental soybean plant, thereby producing a plurality of first progeny plants; then selecting a first progeny plant that is resistant to glufosinate; selfing the first progeny plant, thereby producing a plurality of second progeny plants; and then selecting from the second progeny plants a plant that is resistant to glufosinate. These steps can further include the back-crossing of the first progeny plant or the second progeny plant to the second parental soybean plant or a third parental soybean plant. A soybean crop comprising soybean seeds of the subject invention, or progeny thereof, can then be planted.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating, added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Other breeding methods commonly used for different traits and crops are known in the art. Backcross breeding has been used to transfer genes for a simply inherited, highly heritable trait into a desirable homozygous cultivar or inbred line, which is the recurrent parent. The source of the trait to be transferred is called the donor parent. The resulting plant is expected to have the attributes of the recurrent parent (e.g., cultivar) and the desirable trait transferred from the donor parent. After the initial cross, individuals possessing the phenotype of the donor parent are selected and repeatedly crossed (backcrossed) to the recurrent parent. The resulting parent is expected to have the attributes of the recurrent parent (e.g., cultivar) and the desirable trait transferred from the donor parent.

Likewise an insect resistant/glufosinate-tolerant soybean plant of the subject invention can be transformed with additional transgenes using methods known in the art. Transformation techniques such as *Agrobacterium* transformation, the biolistic transformation (i.e. gene gun), and silicon carbide mediated transformation (i.e. WHISKERS), can be used to introduced additional transgene(s) into the genome of soybean event 9582.814.19.1. Selection and characterization of transgenic plants containing the newly inserted transgenes can be completed to identify plants which contain a stable integrant of the novel transgene in addition to cry1F, cry1Ac, pat genes of the subject invention.

The DNA molecules of the present invention can be used as molecular markers in a marker assisted breeding (MAB) method. DNA molecules of the present invention can be used in methods (such as, AFLP markers, RFLP markers, RAPD markers, SNPs, and SSRs) that identify genetically linked agronomically useful traits, as is known in the art. The insect resistance and herbicide-tolerance traits can be tracked in the progeny of a cross with a soybean plant of the subject invention (or progeny thereof and any other soybean cultivar or variety) using the MAB methods. The DNA molecules are markers for this trait, and MAB methods that are well known in the art can be used to track the herbicide-resistance trait(s) in soybean plants where at least one soybean line of the subject invention, or progeny thereof, was a parent or ancestor. The methods of the present invention can be used to identify any soybean variety having the subject event.

Methods of the subject invention include a method of producing an insect resistant/herbicide-tolerant soybean plant wherein said method comprises breeding with a plant of the subject invention. More specifically, said methods can

US 9,695,441 B2

9

comprise crossing two plants of the subject invention, or one plant of the subject invention and any other plant. Preferred methods further comprise selecting progeny of said cross by analyzing said progeny for an event detectable according to the subject invention and favorable varietal performance (e.g. yield). For example, the subject invention can be used to track the subject event through breeding cycles with plants comprising other desirable traits, such as agronomic traits, disease tolerance or resistance, nematode tolerance or resistance and maturity date. Plants comprising the subject event and the desired trait can be detected, identified, selected, and quickly used in further rounds of breeding, for example. The subject event/trait can also be combined through breeding, and tracked according to the subject invention, with further insect resistant trait(s) and/or with further herbicide tolerance traits. Embodiments of the latter are plants comprising the subject event combined with the aad-12 gene, which confers tolerance to 2,4-dichlorophenoxyacetic acid and pyridyloxyacetate herbicides, or with a gene encoding resistance to the herbicide dicamba.

Thus, the subject invention can be combined with, for example, traits encoding glyphosate resistance (e.g., resistant plant or bacterial EPSPS, GOX, GAT), glufosinate resistance (e.g., pat, bar), acetolactate synthase (ALS)-inhibiting herbicide resistance (e.g., imidazolinones [such as imazethapyr], sulfonyleureas, triazolopyrimidine sulfonanilide, pyrimidinylthiobenzoates, and other chemistries [Csr1, SurA, et al.]), bromoxynil resistance (e.g., Bxn), resistance to inhibitors of HPPD (4-hydroxyphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (e.g., psbA), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (e.g., PPO-I), resistance to phenylurea herbicides (e.g., CYP76B1), dicamba-degrading enzymes (see, e.g., US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes.

Additionally, soybean event 9582.814.19.1 can be combined with one or more additional input (e.g., insect resistance, pathogen resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

Methods to integrate a polynucleotide sequence within a specific chromosomal site of a plant cell via homologous recombination have been described within the art. For instance, site specific integration as described in US Patent Application Publication No. 2009/011188 A1, herein incorporated by reference, describes the use of recombinases or integrases to mediate the introduction of a donor polynucleotide sequence into a chromosomal target. In addition, International Patent Application No. WO 2008/021207, herein incorporated by reference, describes zinc finger mediated-homologous recombination to integrate one or more donor polynucleotide sequences within specific locations of the genome. The use of recombinases such as FLP/FRT as described in U.S. Pat. No. 6,720,475, herein incorporated by reference, or CRE/LOX as described in U.S. Pat. No. 5,658,772, herein incorporated by reference, can be utilized to integrate a polynucleotide sequence into a specific chromosomal site. Finally the use of meganucleases for targeting

10

donor polynucleotides into a specific chromosomal location was described in Puchta et al., PNAS USA 93 (1996) pp. 5055-5060).

Other methods for site specific integration within plant cells are generally known and applicable (Kumar et al., *Trends in Plant Sci.* 6(4) (2001) pp. 155-159). Furthermore, site-specific recombination systems which have been identified in several prokaryotic and lower eukaryotic organisms may be applied to use in plants. Examples of such systems include, but are not limited too; the R/RS recombinase system from the pSR1 plasmid of the yeast *Zygosaccharomyces rouxii* (Araki et al. (1985) J. Mol. Biol. 182: 191-203), and the Gin/gix system of phage Mu (Maeser and Kahlmann (1991) Mol. Gen. Genet. 230: 170-176).

In some embodiments of the present invention, it can be desirable to integrate or stack a new transgene(s) in proximity to an existing transgenic event. The transgenic event can be considered a preferred genomic locus which was selected based on unique characteristics such as single insertion site, normal Mendelian segregation and stable expression, and a superior combination of efficacy, including herbicide tolerance and agronomic performance in and across multiple environmental locations. The newly integrated transgenes should maintain the transgene expression characteristics of the existing transformants. Moreover, the development of assays for the detection and confirmation of the newly integrated event would be overcome as the genomic flanking sequences and chromosomal location of the newly integrated event are already identified. Finally, the integration of a new transgene into a specific chromosomal location which is linked to an existing transgene would expedite the introgression of the transgenes into other genetic backgrounds by sexual out-crossing using conventional breeding methods.

In some embodiments of the present invention, it can be desirable to excise polynucleotide sequences from a transgenic event. For instance transgene excision as described in Provisional U.S. Patent Application No. 61/297,628, herein incorporated by reference, describes the use of zinc finger nucleases to remove a polynucleotide sequence, consisting of a gene expression cassette, from a chromosomally integrated transgenic event. The polynucleotide sequence which is removed can be a selectable marker. Upon excision and removal of a polynucleotide sequence the modified transgenic event can be retargeted by the insertion of a polynucleotide sequence. The excision of a polynucleotide sequence and subsequent retargeting of the modified transgenic event provides advantages such as re-use of a selectable marker or the ability to overcome unintended changes to the plant transcriptome which results from the expression of specific genes.

The subject invention discloses herein a specific site on chromosome 02 in the soybean genome that is excellent for insertion of heterologous nucleic acids. Thus, the subject invention provides methods to introduce heterologous nucleic acids of interest into this pre-established target site or in the vicinity of this target site. The subject invention also encompasses a soybean seed and/or a soybean plant comprising any heterologous nucleotide sequence inserted at the disclosed target site or in the general vicinity of such site. One option to accomplish such targeted integration is to excise and/or substitute a different insert in place of the pat expression cassette exemplified herein. In this general regard, targeted homologous recombination, for example and without limitation, can be used according to the subject invention.

US 9,695,441 B2

11

As used herein gene, event or trait “stacking” is combining desired traits into one transgenic line. Plant breeders stack transgenic traits by making crosses between parents that each have a desired trait and then identifying offspring that have both of these desired traits. Another way to stack genes is by transferring two or more genes into the cell nucleus of a plant at the same time during transformation. Another way to stack genes is by re-transforming a transgenic plant with another gene of interest. For example, gene stacking can be used to combine two or more different traits, including for example, two or more different insect traits, insect resistance trait(s) and disease resistance trait(s), two or more herbicide resistance traits, and/or insect resistance trait(s) and herbicide resistant trait(s). The use of a selectable marker in addition to a gene of interest can also be considered gene stacking.

“Homologous recombination” refers to a reaction between any pair of nucleotide sequences having corresponding sites containing a similar nucleotide sequence through which the two nucleotide sequences can interact (recombine) to form a new, recombinant DNA sequence. The sites of similar nucleotide sequence are each referred to herein as a “homology sequence.” Generally, the frequency of homologous recombination increases as the length of the homology sequence increases. Thus, while homologous recombination can occur between two nucleotide sequences that are less than identical, the recombination frequency (or efficiency) declines as the divergence between the two sequences increases. Recombination may be accomplished using one homology sequence on each of the donor and target molecules, thereby generating a “single-crossover” recombination product. Alternatively, two homology sequences may be placed on each of the target and donor nucleotide sequences. Recombination between two homology sequences on the donor with two homology sequences on the target generates a “double-crossover” recombination product. If the homology sequences on the donor molecule flank a sequence that is to be manipulated (e.g., a sequence of interest), the double-crossover recombination with the target molecule will result in a recombination product wherein the sequence of interest replaces a DNA sequence that was originally between the homology sequences on the target molecule. The exchange of DNA sequence between the target and donor through a double-crossover recombination event is termed “sequence replacement.”

A preferred plant, or a seed, of the subject invention comprises in its genome operative cry1F v3, cry1Ac synpro and pat v6 nucleotide sequences, as identified herein, together with at least 20-500 or more contiguous flanking nucleotides on both sides of the insert, as identified herein. Unless indicated otherwise, reference to flanking sequences refers to those identified with respect to SEQ ID NOS: 1 and 2. All or part of these flanking sequences could be expected to be transferred to progeny that receives the inserted DNA as a result of a sexual cross of a parental line that includes the event.

The subject invention includes tissue cultures of regenerable cells of a plant of the subject invention. Also included is a plant regenerated from such tissue culture, particularly where said plant is capable of expressing all the morphological and physiological properties of an exemplified variety. Preferred plants of the subject invention have all the physiological and morphological characteristics of a plant grown from the deposited seed. This invention further comprises progeny of such seed and seed possessing the quality traits of interest.

12

As used herein, a “line” is a group of plants that display little or no genetic variation between individuals for at least one trait. Such lines may be created by several generations of self-pollination and selection, or vegetative propagation from a single parent using tissue or cell culture techniques.

As used herein, the terms “cultivar” and “variety” are synonymous and refer to a line which is used for commercial production.

“Stability” or “stable” means that with respect to the given component, the component is maintained from generation to generation and, preferably, at least three generations.

“Commercial Utility” is defined as having good plant vigor and high fertility, such that the crop can be produced by farmers using conventional farming equipment, and the oil with the described components can be extracted from the seed using conventional crushing and extraction equipment.

“Agronomically elite” means that a line has desirable agronomic characteristics such as yield, maturity, disease resistance, and the like, in addition to the insect resistance and herbicide tolerance due to the subject event(s). Any and all of these agronomic characteristics and data points can be used to identify such plants, either as a point or at either end or both ends of a range of characteristics used to define such plants.

As one skilled in the art will recognize in light of this disclosure, preferred embodiments of detection kits, for example, can include probes and/or primers directed to and/or comprising “junction sequences” or “transition sequences” (where the soybean genomic flanking sequence meets the insert sequence). For example, this includes a polynucleotide probes, primers, and/or amplicons designed to identify one or both junction sequences (where the insert meets the flanking sequence), as indicated in the Table above. One common design is to have one primer that hybridizes in the flanking region, and one primer that hybridizes in the insert. Such primers are often each about at least ~15 residues in length. With this arrangement, the primers can be used to generate/amplify a detectable amplicon that indicates the presence of an event of the subject invention. These primers can be used to generate an amplicon that spans (and includes) a junction sequence as indicated above.

The primer(s) “touching down” in the flanking sequence is typically not designed to hybridize beyond about 1200 bases or so beyond the junction. Thus, typical flanking primers would be designed to comprise at least 15 residues of either strand within 1200 bases into the flanking sequences from the beginning of the insert. That is, primers comprising a sequence of an appropriate size from (or hybridizing to) base pairs 800 to 1400 of SEQ ID NO:14 and/or base pairs 13,897 to 14,497 of SEQ ID NO:14 are within the scope of the subject invention. Insert primers can likewise be designed anywhere on the, but base pairs 1400 to 2000 of SEQ ID NO:14 and/or base pairs 13,297 to 13,896 of SEQ ID NO:14, and can be used, for example, non-exclusively for such primer design.

One skilled in the art will also recognize that primers and probes can be designed to hybridize, under a range of standard hybridization and/or PCR conditions wherein the primer or probe is not perfectly complementary to the exemplified sequence. That is, some degree of mismatch can be tolerated. For an approximately 20 nucleotide primer, for example, typically one or two or so nucleotides do not need to bind with the opposite strand if the mismatched base is internal or on the end of the primer that is opposite the amplicon. Various appropriate hybridization conditions are



US 9,695,441 B2

13

provided below. Synthetic nucleotide analogs, such as inosine, can also be used in probes. Peptide nucleic acid (PNA) probes, as well as DNA and RNA probes, can also be used. What is important is that such probes and primers are diagnostic for (able to uniquely identify and distinguish) the presence of an event of the subject invention.

It should be noted that errors in PCR amplification can occur which might result in minor sequencing errors, for example. That is, unless otherwise indicated, the sequences listed herein were determined by generating long amplicons from soybean genomic DNAs, and then cloning and sequencing the amplicons. It is not unusual to find slight differences and minor discrepancies in sequences generated and determined in this manner, given the many rounds of amplification that are necessary to generate enough amplicon for sequencing from genomic DNAs. One skilled in the art should recognize and be put on notice that any adjustments needed due to these types of common sequencing errors or discrepancies are within the scope of the subject invention.

It should also be noted that it is not uncommon for some genomic sequence to be deleted, for example, when a sequence is inserted during the creation of an event. Thus, some differences can also appear between the subject flanking sequences and genomic sequences listed in GENBANK, for example.

Components of the DNA sequence "insert" are illustrated in the Figures and are discussed in more detail below in the Examples. The DNA polynucleotide sequences of these components, or fragments thereof, can be used as DNA primers or probes in the methods of the present invention.

In some embodiments of the invention, compositions and methods are provided for detecting the presence of the transgene/genomic insertion region, in plants and seeds and the like, from a soybean plant. DNA sequences are provided that comprise the subject 5' transgene/genomic insertion region junction sequence provided herein (between base pairs 800 to 1400 of SEQ ID NO:14), segments thereof, and complements of the exemplified sequences and any segments thereof. DNA sequences are provided that comprise the subject 3' transgene/genomic insertion region junction sequence provided herein (between base pairs 13,897 to 14,497 of SEQ ID NO:14), segments thereof, and complements of the exemplified sequences and any segments thereof. The insertion region junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the soybean cell flanking the insertion site. Such sequences can be diagnostic for the given event.

Based on these insert and border sequences, event-specific primers can be generated. PCR analysis demonstrated that soybean lines of the subject invention can be identified in different soybean genotypes by analysis of the PCR amplicons generated with these event-specific primer sets. These and other related procedures can be used to uniquely identify these soybean lines. Thus, PCR amplicons derived from such primer pairs are unique and can be used to identify these soybean lines.

In some embodiments, DNA sequences that comprise a contiguous fragment of the novel transgene/genomic insertion region are an aspect of this invention. Included are DNA sequences that comprise a sufficient length of polynucleotides of transgene insert sequence and a sufficient length of polynucleotides of soybean genomic sequence from one or more of the three aforementioned soybean plants and/or

14

sequences that are useful as primer sequences for the production of an amplicon product diagnostic for one or more of these soybean plants.

Related embodiments pertain to DNA sequences that comprise at least 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or more contiguous nucleotides of a transgene portion of a DNA sequence identified herein (such as SEQ ID NO: 1 and segments thereof), or complements thereof, and a similar length of flanking soybean DNA sequence from these sequences, or complements thereof. Such sequences are useful as DNA primers in DNA amplification methods. The amplicons produced using these primers are diagnostic for any of the soybean events referred to herein. Therefore, the invention also includes the amplicons produced by such DNA primers and homologous primers.

This invention also includes methods of detecting the presence of DNA, in a sample, that corresponds to the soybean event referred to herein. Such methods can comprise: (a) contacting the sample comprising DNA with a primer set that, when used in a nucleic acid amplification reaction with DNA from at least one of these soybean events, produces an amplicon that is diagnostic for said event(s); (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon.

Further detection methods of the subject invention include a method of detecting the presence of a DNA, in a sample, corresponding to said event, wherein said method comprises: (a) contacting the sample comprising DNA with a probe that hybridizes under stringent hybridization conditions with DNA from at least one of said soybean events and which does not hybridize under the stringent hybridization conditions with a control soybean plant (non-event-of-interest DNA); (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.

In still further embodiments, the subject invention includes methods of producing a soybean plant comprising soybean event 9582.814.19.1 of the subject invention, wherein said method comprises the steps of: (a) sexually crossing a first parental soybean line (comprising an expression cassettes of the present invention, which confers glufosinate tolerance to plants of said line) and a second parental soybean line (that lacks this herbicide tolerance trait) thereby producing a plurality of progeny plants; and (b) selecting a progeny plant by the use of molecular markers. Such methods may optionally comprise the further step of back-crossing the progeny plant to the second parental soybean line to producing a true-breeding soybean plant that comprises the insect resistant and glufosinate tolerant trait.

According to another aspect of the invention, methods of determining the zygosity of progeny of a cross with said event is provided. Said methods can comprise contacting a sample, comprising soybean DNA, with a primer set of the subject invention. Said primers, when used in a nucleic-acid amplification reaction with genomic DNA from at least one of said soybean events, produces a first amplicon that is diagnostic for at least one of said soybean events. Such methods further comprise performing a nucleic acid amplification reaction, thereby producing the first amplicon; detecting the first amplicon; and contacting the sample comprising soybean DNA with a second primer set (said second primer set, when used in a nucleic-acid amplification reaction with genomic DNA from soybean plants, produces a second amplicon comprising the native soybean genomic DNA homologous to the soybean genomic region); and

US 9,695,441 B2

15

performing a nucleic acid amplification reaction, thereby producing the second amplicon. The methods further comprise detecting the second amplicon, and comparing the first and second amplicons in a sample, wherein the presence of both amplicons indicates that the sample is heterozygous for the transgene insertion.

DNA detection kits can be developed using the compositions disclosed herein and methods well known in the art of DNA detection. The kits are useful for identification of the subject soybean event DNA in a sample and can be applied to methods for breeding soybean plants containing this DNA. The kits contain DNA sequences homologous or complementary to the amplicons, for example, disclosed herein, or to DNA sequences homologous or complementary to DNA contained in the transgene genetic elements of the subject events. These DNA sequences can be used in DNA amplification reactions or as probes in a DNA hybridization method. The kits may also contain the reagents and materials necessary for the performance of the detection method.

A "probe" is an isolated nucleic acid molecule to which is attached a conventional detectable label or reporter molecule (such as a radioactive isotope, ligand, chemiluminescent agent, or enzyme). Such a probe is complementary to a strand of a target nucleic acid, in the case of the present invention, to a strand of genomic DNA from one of said soybean events, whether from a soybean plant or from a sample that includes DNA from the event. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

"Primers" are isolated/synthesized nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs of the present invention refer to their use for amplification of a target nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

Probes and primers are generally 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334,

16

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Methods for preparing and using probes and primers are described, for example, in

Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989. PCR-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose.

Primers and probes based on the flanking DNA and insert sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed sequences by conventional methods, e.g., by re-cloning and sequencing such sequences.

The nucleic acid probes and primers of the present invention hybridize under stringent conditions to a target DNA sequence. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. Nucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit "complete complementarity" when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Conventional stringency conditions are described by Sambrook et al., 1989. Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.



US 9,695,441 B2

17

As used herein, a substantially homologous sequence is a nucleic acid sequence that will specifically hybridize to the complement of the nucleic acid sequence to which it is being compared under high stringency conditions. The term "stringent conditions" is functionally defined with regard to the hybridization of a nucleic-acid probe to a target nucleic acid (i.e., to a particular nucleic-acid sequence of interest) by the specific hybridization procedure discussed in Sambrook et al., 1989, at 9.52-9.55. See also, Sambrook et al., 1989 at 9.47-9.52 and 9.56-9.58. Accordingly, the nucleotide sequences of the invention may be used for their ability to selectively form duplex molecules with complementary stretches of DNA fragments.

Depending on the application envisioned, one can use varying conditions of hybridization to achieve varying degrees of selectivity of probe towards target sequence. For applications requiring high selectivity, one will typically employ relatively stringent conditions to form the hybrids, e.g., one will select relatively low salt and/or high temperature conditions, such as provided by about 0.02 M to about 0.15 M NaCl at temperatures of about 50° C. to about 70° C. Stringent conditions, for example, could involve washing the hybridization filter at least twice with high-stringency wash buffer (0.2×SSC, 0.1% SDS, 65° C.). Appropriate stringency conditions which promote DNA hybridization, for example, 6.0× sodium chloride/sodium citrate (SSC) at about 45° C., followed by a wash of 2.0×SSC at 50° C. are known to those skilled in the art. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0×SSC at 50° C. to a high stringency of about 0.2×SSC at 50° C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22° C., to high stringency conditions at about 65° C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed. Such selective conditions tolerate little, if any, mismatch between the probe and the template or target strand. Detection of DNA sequences via hybridization is well-known to those of skill in the art, and the teachings of U.S. Pat. Nos. 4,965,188 and 5,176,995 are exemplary of the methods of hybridization analyses.

In a particularly preferred embodiment, a nucleic acid of the present invention will specifically hybridize to one or more of the primers (or amplicons or other sequences) exemplified or suggested herein, including complements and fragments thereof, under high stringency conditions. In one aspect of the present invention, a marker nucleic acid molecule of the present invention has the nucleic acid sequence as set forth herein in one of the exemplified sequences, or complements and/or fragments thereof.

In another aspect of the present invention, a marker nucleic acid molecule of the present invention shares between 80% and 100% or 90% and 100% sequence identity with such nucleic acid sequences. In a further aspect of the present invention, a marker nucleic acid molecule of the present invention shares between 95% and 100% sequence identity with such sequence. Such sequences may be used as markers in plant breeding methods to identify the progeny of genetic crosses. The hybridization of the probe to the target DNA molecule can be detected by any number of methods known to those skilled in the art, these can include, but are not limited to, fluorescent tags, radioactive tags, antibody based tags, and chemiluminescent tags.

Regarding the amplification of a target nucleic acid sequence (e.g., by PCR) using a particular amplification primer pair, "stringent conditions" are conditions that permit

18

the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product, the amplicon.

The term "specific for (a target sequence)" indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

As used herein, "amplified DNA" or "amplicon" refers to the product of nucleic-acid amplification of a target nucleic acid sequence that is part of a nucleic acid template. For example, to determine whether the soybean plant resulting from a sexual cross contains transgenic event genomic DNA from the soybean plant of the present invention, DNA extracted from a soybean plant tissue sample may be subjected to nucleic acid amplification method using a primer pair that includes a primer derived from flanking sequence in the genome of the plant adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA. The amplicon is of a length and has a sequence that is also diagnostic for the event. The amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair, and/or the combined length of the primer pairs plus about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, 293, 294, 295, 296, 297, 298, 299, 300, 301, 302, 303, 304, 305, 306, 307, 308, 309, 310, 311, 312, 313, 314, 315, 316, 317, 318, 319, 320, 321, 322, 323, 324, 325, 326, 327, 328, 329, 330, 331, 332, 333, 334, 335, 336, 337, 338, 339, 340, 341, 342, 343, 344, 345, 346, 347, 348, 349, 350, 351, 352, 353, 354, 355, 356, 357, 358, 359, 360, 361, 362, 363, 364, 365, 366, 367, 368, 369, 370, 371, 372, 373, 374, 375, 376, 377, 378, 379, 380, 381, 382, 383, 384, 385, 386, 387, 388, 389, 390, 391, 392, 393, 394, 395, 396, 397, 398, 399, 400, 401, 402, 403, 404, 405, 406, 407, 408, 409, 410, 411, 412, 413, 414, 415, 416, 417, 418, 419, 420, 421, 422, 423, 424, 425, 426, 427, 428, 429, 430, 431, 432, 433, 434, 435, 436, 437, 438, 439, 440, 441, 442, 443, 444, 445, 446, 447, 448, 449, 450, 451, 452, 453, 454, 455, 456, 457, 458, 459, 460, 461, 462, 463, 464, 465, 466, 467, 468, 469, 470, 471, 472, 473, 474, 475, 476, 477, 478, 479, 480, 481, 482, 483, 484, 485, 486, 487, 488, 489, 490, 491, 492, 493, 494, 495, 496, 497, 498, 499, or 500, 750, 1000, 1250, 1500, 1750, 2000, or more nucleotide base pairs

US 9,695,441 B2

19

(plus or minus any of the increments listed above). Alternatively, a primer pair can be derived from flanking sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence. A member of a primer pair derived from the plant genomic sequence may be located a distance from the inserted DNA sequence. This distance can range from one nucleotide base pair up to about twenty thousand nucleotide base pairs. The use of the term "amplicon" specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

Nucleic-acid amplification can be accomplished by any of the various nucleic-acid amplification methods known in the art, including the polymerase chain reaction (PCR). A variety of amplification methods are known in the art and are described, *inter alia*, in U.S. Pat. No. 4,683,195 and U.S. Pat. No. 4,683,202. PCR amplification methods have been developed to amplify up to 22 kb of genomic DNA. These methods as well as other methods known in the art of DNA amplification may be used in the practice of the present invention. The sequence of the heterologous transgene DNA insert or flanking genomic sequence from a subject soybean event can be verified (and corrected if necessary) by amplifying such sequences from the event using primers derived from the sequences provided herein followed by standard DNA sequencing of the PCR amplicon or of the cloned DNA.

The amplicon produced by these methods may be detected by a plurality of techniques. Agarose gel electrophoresis and staining with ethidium bromide is a common well known method of detecting DNA amplicons. Another such method is Genetic Bit Analysis where an DNA oligonucleotide is designed which overlaps both the adjacent flanking genomic DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking genomic sequence), a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Another method is the Pyrosequencing technique as described by Winge (Innov. Pharma. Tech. 00:18-24, 2000). In this method an oligonucleotide is designed that overlaps the adjacent genomic DNA and insert DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking genomic sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. DNTPs are added individually and the incorporation results in a light signal that is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Fluorescence Polarization is another method that can be used to detect an amplicon of the present invention. Following this method, an oligonucleotide is designed which overlaps the genomic flanking and inserted DNA junction. The oligonucleotide is hybridized to single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking genomic DNA sequence) and incubated in the presence of a DNA poly-

20

merase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single base extension.

TAQMAN® (PE Applied Biosystems, Foster City, Calif.) is a method of detecting and quantifying the presence of a DNA sequence. Briefly, a FRET oligonucleotide probe is designed that overlaps the genomic flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. During specific amplification, Taq DNA polymerase cleaves and releases the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

Molecular Beacons have been described for use in sequence detection. Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking genomic and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal indicates the presence of the flanking genomic/transgene insert sequence due to successful amplification and hybridization.

Having disclosed a location in the soybean genome that is excellent for an insertion, the subject invention also comprises a soybean seed and/or a soybean plant comprising at least one non-soybean event 9582.814.19.1 insert in the general vicinity of this genomic location. One option is to substitute a different insert in place of the one from soybean event pDAB9582.814.19.1 exemplified herein. In these general regards, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (US 20030232410). Thus, the subject invention includes plants and plant cells comprising a heterologous insert (in place of or with multi-copies of the cry1F cry1Ac, or pat genes), flanked by all or a recognizable part of the flanking sequences identified herein (bp 1-1400 of SEQ ID NO:1 and bp 153-1550 of SEQ ID NO:2). An additional copy (or additional copies) of a cry1F, cry1Ac, or pat could also be targeted for insertion in this/these manner(s).

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification

The following examples are included to illustrate procedures for practicing the invention and to demonstrate certain preferred embodiments of the invention. These examples should not be construed as limiting. It should be appreciated by those of skill in the art that the techniques disclosed in the following examples represent specific approaches used to illustrate preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in these specific

US 9,695,441 B2

21

embodiments while still obtaining like or similar results without departing from the spirit and scope of the invention. Unless otherwise indicated, all percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

The following abbreviations are used unless otherwise indicated.

bp base pair

° C. degrees Celsius

DNA deoxyribonucleic acid

EDTA ethylenediaminetetraacetic acid

kb kilobase

µg microgram

µL microliter

mL milliliter

M molar mass

PCR polymerase chain reaction

PTU plant transcription unit

SDS sodium dodecyl sulfate

SSC a buffer solution containing a mixture of sodium chloride and sodium citrate, pH 7.0

TBE a buffer solution containing a mixture of Tris base, boric acid and EDTA, pH 8.3

Embodiments of the present invention are further defined in the following Examples. It should be understood that these Examples are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the embodiments of the invention to adapt it to various usages and conditions. Thus, various modifications of the embodiments of the invention, in addition to those shown and described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

## EXAMPLES

### Example 1: Transformation and Selection of the Cry1F and Cry1Ac Soybean Event pDAB9582.814.19.1

Transgenic soybean (*Glycine max*) containing the soybean event pDAB9582.814.19.1 was generated through *Agrobacterium*-mediated transformation of soybean cotyledonary node explants. The disarmed *Agrobacterium* strain EHA101 (Hood et al., 1993), carrying the binary vector pDAB9582 (FIG. 1) containing the selectable marker, *pat* v6, and the genes of interest, *cry1F* v3 and *cry1Ac* synpro, within the T-strand DNA region, was used to initiate transformation. The DNA sequence for pDAB9582 is given in SEQ ID NO:3, which is annotated below in Table 1.

TABLE 1

Gene elements located on pDAB9582.		
bp (SEQ ID NO: 3)	Construct element	Reference
272-1593	AtUbi10 Promoter	Callis, et al., (1990) <i>J. Biol. Chem.</i> , 265: 12486-12493
1602-5048	Cry1F	Referenced above
5151-5607	ORF23 3'UTR	U.S. Pat. No. 5,428,147

22

TABLE 1-continued

Gene elements located on pDAB9582.		
bp (SEQ ID NO: 3)	Construct element	Reference
5671-6187	CsVMV Promoter	Verdaguer et al., (1996) <i>Plant Mol. Biol.</i> , 31: 1129-1139
6197-9667	Cry 1AC	Referenced above
9701-10157	ORF23 3'UTR	U.S. Pat. No. 5,428,147
10272-10788	CsVMV Promoter	Verdaguer et al., (1996) <i>Plant Mol. Biol.</i> , 31: 1129-1139
10796-11347	PAT	Wohlleben et al., (1988) <i>Gene</i> 70: 25-37
11450-12153	ORF1 3'UTR	Huang et al., (1990) <i>J. Bacteriol.</i> 172: 1814-1822

*Agrobacterium*-mediated transformation was carried out using a modified procedure of Zeng et al. (2004). Briefly, soybean seeds (cv Maverick) were germinated on basal media and cotyledonary nodes were isolated and infected with *Agrobacterium*. Shoot initiation, shoot elongation, and rooting media were supplemented with cefotaxime, timentin and vancomycin for removal of *Agrobacterium*. Glufosinate selection was employed to inhibit the growth of non-transformed shoots. Selected shoots were transferred to rooting medium for root development and then transferred to soil mix for acclimatization of plantlets.

Terminal leaflets of selected plantlets were leaf painted with glufosinate to screen for putative transformants. The screened plantlets were transferred to the greenhouse, allowed to acclimate and then leaf-painted with glufosinate to reconfirm tolerance and deemed to be putative transformants. The screened plants were sampled and molecular analyses for the confirmation of the selectable marker gene and/or the gene of interest were carried out. T<sub>0</sub> plants were allowed to self fertilize in the greenhouse to give rise to T<sub>1</sub> seed.

This event, soybean event pDAB9582.814.19.1, was generated from an independent transformed isolate. The T<sub>1</sub> plants were backcrossed and introgressed into elite varieties over subsequent generations. The event was selected based on its unique characteristics such as single insertion site, normal Mendelian segregation, stable expression, and a superior combination of efficacy, including herbicide tolerance and agronomic performance. The following examples contain the data which were used to characterize soybean event pDAB9582.814.19.1.

### Example 2: Characterization of Protein Expression in Soybean Event pDAB9582.814.19.1

The biochemical properties of the recombinant Cry1F, Cry1Ac, and PAT proteins expressed in soybean event 9582.814.19.1 were characterized. Quantitative enzyme-linked immunosorbent assay (ELISA) is a biochemical assay known within the art that can be used to characterize the biochemical properties of the proteins and confirm expression of these proteins in soybean event 9582.814.19.1.

#### Example 2.1: Expression of the PAT, Cry1F, and Cry1Ac Protein in Plant Tissues

Samples of soybean tissues were isolated from the test plants and prepared for expression analysis. The PAT protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST) containing 0.5% Bovine Serum Albumin (BSA). The plant tissue was centrifuged; the aqueous supernatant

US 9,695,441 B2

23

was collected, diluted with appropriate buffer as necessary, and analyzed using an PAT ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Envirologix, Portland, Me.). This assay measured the expressed PAT protein.

The Cry1F protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST). The plant tissue was centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer as necessary, and analyzed using an Cry1F ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Strategic Diagnostics Inc., Newark, Del.). This assay measured the expressed Cry1F protein.

The Cry1Ac protein was extracted from soybean plant tissues with a phosphate buffered saline solution containing the detergent Tween-20 (PBST) containing 0.5% Bovine Serum Albumin (BSA). The plant tissue was centrifuged; the aqueous supernatant was collected, diluted with appropriate buffer as necessary, and analyzed using an Cry1Ac ELISA kit in a sandwich format. The kit was used following the manufacturer's suggested protocol (Strategic Diagnostics Inc., Newark, Del.). This assay measured the Cry1Ac protein.

Detection analysis was performed to investigate the expression stability and inheritability both vertically (between generations) and horizontally (between lineages within a generation) in soybean event pDAB9582.814.19.1.

#### Example 2.2: Expression of the PAT, Cry1F, and Cry1Ac Protein in Plant Tissues

Levels of Cry1F, Cry1Ac and PAT proteins were determined in Soybean Event 9582.814.19.1. The soluble, extractable proteins were measured using a quantitative enzyme-linked immunosorbent assay (ELISA) method from soybean leaf tissue. From T<sub>2</sub> to T<sub>6</sub> generations Soybean Events 9582.814.19.1, expression was stable (not segregating) and consistent across all lineages. Table 2 lists the mean expression level of the transgenic proteins in soybean event 9582.814.19.1.

24

TABLE 2

Mean expression level of different transgenic proteins in soybean event pDAB9582.814.19.1.

Expression Level of Different Proteins (ng/cm<sup>2</sup>)

Event	Cry1F	Cry1 Ac	PAT
Soybean event pDAB9582.814.19.1	133	17.4	12

#### Example 3: Cloning and Characterization of DNA Sequence in the Insert and the Flanking Border Regions of Soybean Event pDAB9582.814.19.1

To characterize and describe the genomic insertion site, the sequence of the flanking genomic T-DNA border regions of soybean event pDAB9582.814.19.1 were determined. Genomic sequence of soybean event pDAB9582.814.19.1 was confirmed, comprising 1400 bp of 5' flanking border sequence (SEQ ID NO:1) and 1398 bp of 3' flanking border sequence (SEQ ID NO:2). PCR amplification based on the soybean event pDAB9582.814.19.1 border sequences validated that the border regions were of soybean origin and that the junction regions are unique sequences for soybean event pDAB9582.814.19.1. The junction regions could be used for event-specific identification of soybean event pDAB9582.814.19.1. In addition, the T-strand insertion site was characterized by amplifying a genomic fragment corresponding to the region of the identified flanking border sequences from the genome of untransformed soybean. Comparison of soybean event pDAB9582.814.19.1 with the untransformed genomic sequence revealed that a deletion of about 57 bp from the original locus resulted during the T-strand integration. Overall, the characterization of the insert and border sequence of soybean event pDAB9582.814.19.1 indicated that an intact copy of the T-strand from pDAB9582 was present in the soybean genome.

TABLE 3

List of primers and their sequences used in the confirmation of soybean genomic DNA in soybean event pDAB9582.814.19.1

SEQ ID NO:	Primer Name	Size (bp)	Sequence (5' to 3')	Purpose
SEQ ID NO: 4	81419_FW3	30	TTTCTCCTATCCGTC AAATAAATCTGCTCC	confirmation of 5' border genomic DNA, used with AtUbi10RV1 or RV2; with 5' IREnd-01 or 5' IREnd-02
SEQ ID NO: 5	81419_RV1	27	GGGTGATTTGGTGCC AAAAGTTATGTT	confirmation of 3' border genomic DNA, used with 3' PATEnd05 or 3' PATEnd06
SEQ ID NO: 6	81419_RV2	24	TGGAGGGTCATATCG CAAAAGACT	confirmation of 3' border genomic DNA, used with 3' PATEnd05 or 3' PATEnd06
SEQ ID NO: 7	81419_RV3	24	GTTCTGCGTCGTGGA GGGTCATAT	confirmation of 3' border genomic DNA, used with 3' PATEnd05 or 3' PATEnd06

US 9,695,441 B2

25

26

TABLE 3-continued

List of primers and their sequences used in the confirmation of soybean genomic DNA in soybean event pDAB9582.814.19.1			
SEQ ID NO:	Primer Name	Size (bp)	Sequence (5' to 3') Purpose
SEQ ID NO: 8	IREnd-0129	CGAGCTTTCTAATT CAAACATATTTCGGGC	confirmation of 5' border genomic DNA, used with 81419_FW3
SEQ ID NO: 9	5'IREnd-0230	TCCTAGATCATCAGT TCATACAAACCTCCA	confirmation of 5' border genomic DNA, used with 81419_FW3
SEQ ID NO: 10	AtUbi10 RV1	29 CGGTCCTAGATCATC AGTTCATACAAACC	confirmation of 5' border genomic DNA, used with 81419_FW3
SEQ ID NO: 11	AtUbi10 RV2	28 CACTCGTGTTCAGTC CAATGACCAATAA	confirmation of 5' border genomic DNA, used with 81419_FW3
SEQ ID NO: 12	3'PATE nd05	20 GCTCCTCCAAGGCCA GTTAG	confirmation of 3' border genomic DNA, used with 81419_RV1, RV2 or RV3
SEQ ID NO: 13	3'PATE nd06	20 CCAGTTAGGCCAGTT ACCCA	confirmation of 3' border genomic DNA, used with 81419_RV1, RV2 or RV3

TABLE 4

Conditions for standard PCR amplification of the border regions and event-specific sequences in soybean event pDAB9582.814.19.1.						
Target Sequence	Primer Set	PCR Mixture	Pre-denaturation (° C./min)	Denaturation (° C./sec.)	Extension (° C./min:sec)	Final Extension (° C./min)
5' border	81419_FW3/ AtUbi10RV1	D	95/3	98/10	68/4:00 32 cycles	72/10
5' border	81419_FW3/ 5'IREnd-01	D	95/3	98/10	68/4:00 32 cycles	72/10
3' border	3'PATEnd05/ 81419_RV2	D	95/3	98/10	68/4:00 35 cycles	72/10
3' border	3'PATEnd05/ 81419_RV3	D	95/3	98/10	68/4:00 35 cycles	72/10
3' border	3'PATEnd06/ 81419_RV2	D	95/3	98/10	68/4:00 35 cycles	72/10
3' border	3'PATEnd06/ 81419_RV3	D	95/3	98/10	68/4:00 32 cycles	72/10
Across the insert locus	81419_FW3/ 81419_RV3	D	95/3	98/10	68/4:00 32 cycles	72/10

TABLE 5

PCR mixture for standard PCR amplification of the border regions and event specific sequences in soybean event pDAB9582.814.19.1.			
Reagent	1 x reaction (μL)	Reagent	1 x reaction (μL)
PCR Mixture A		PCR Mixture B	
H2O	0.8	H2O	14.6
ACCPRIIME PFX SUPERMIX	20	10X LA TAQ BUFFER	2
—	—	MgCl2 (25 mM)	0.6
—	—	dNTP (2.5 uM)	1.6
10 uM primer	0.2	10 uM primer	0.1

TABLE 5-continued

PCR mixture for standard PCR amplification of the border regions and event specific sequences in soybean event pDAB9582.814.19.1.			
Reagent	1 x reaction (μL)	Reagent	1 x reaction (μL)
PCR Mixture C		PCR Mixture D	
gDNA digestion	1	gDNA digestion	1
—	—	LA TAQ (5U/ul)	0.1
rxn vol:	22	rxn vol:	20
H2O	28	H2O	11.6
10X PCR buffer II (Mg-plus)	5	10X PCR buffer II (Mg-plus)	2



US 9,695,441 B2

27

TABLE 5-continued

PCR mixture for standard PCR amplification of the border regions and event specific sequences in soybean event pDAB9582.814.19.1.			
Reagent	1 x reaction (μL)	Reagent	1 x reaction (μL)
MgCl <sub>2</sub> [25 mM]	1.5	MgCl <sub>2</sub> [25 mM]	0.6
dNTP [2.5 mM]	8	dNTP [2.5 mM]	3.2
Adaptor PCR primer (10 μM)	1	primer1 (10 μM)	0.4
GOI nested primer (10 μM)	1	primer2 (10 μM)	0.4
DNA binded Beads	5	DNA Template	0.2
LA TAQ (5U/ul)	0.5	LA TAQ (5U/ul)	1.6
rxn vol:	50	rxn vol:	20

#### Example 3.1: Confirmation of Soybean Genomic Sequences

The 5' and 3' flanking borders aligned to a *Glycine max* whole genome shotgun sequence from chromosome 02, indicating that the transgene of soybean event pDAB9582.814.19.1 was inserted in soybean genome chromosome 02. To confirm the insertion site of soybean event pDAB9582.814.19.1 from the soybean genome, PCR was carried out with different pairs of primers (FIG. 2, Table 3, Table 4, and Table 5). Genomic DNA from soybean event pDAB9582.814.19.1 and other transgenic or non-transgenic soybean lines was used as a template. To confirm that the 5' border sequences are correct a primer designed to bind to the At Ubi10 promoter gene element, for example AtUbi10RV1, and a primer designed to bind to the cloned 5' end border on soybean genome chromosome 02, primer designated 81419\_FW3, were used for amplifying the DNA segment that spans the AtUbi10 promoter gene element to 5' end border sequence. Similarly, for confirmation of the cloned 3' border sequence a pat specific primer, for example 3'PAT-End05, and three primers designed according to the cloned 3' end border sequence, designated 81419\_RV1, 81419\_RV2 and 81419\_RV3, were used for amplifying DNA segments that span the pat gene to 3' border sequence. DNA fragments with expected sizes were amplified only from the genomic DNA of soybean event pDAB9582.814.19.1 with each primer pair, but not from DNA samples from other transgenic soybean lines or the non-transgenic control. The results indicate that the cloned 5' and 3' border sequences are the flanking border sequences of the T-strand insert for soybean event pDAB9582.814.19.1.

To further confirm the DNA insertion in the soybean genome, a PCR amplification spanning the soybean border sequences was completed on genomic DNA which did not contain the T-strand insert for soybean event pDAB9582.814.19.1. Primer 81419\_FW3, designed according to the 5' end border sequence, and one primer 81419-RV3, designed for the 3' end border sequence, were used to amplify DNA segments which contained the locus where the pDAB9582 T-strand integrated. As expected, PCR amplification completed with the primer pair of 81419\_FW3 and 81419\_RV3 produced an approximately a 1.5 kb DNA fragment from all the other soybean control lines but not pDAB9582.814.19.1. Aligning the identified 5' and 3' border sequences of soybean event pDAB9582.814.19.1 with a *Glycine max* whole genome shotgun sequence from chromosome 02 revealed about 57 bp deletion from the original locus. (FIG. 3). These results demonstrated that the trans-

28

gene of soybean event pDAB8294 was inserted into the site of soybean genome chromosome 02.

#### Example 4: Soybean Event pDAB9582.814.19.1 Characterization Via Southern Blot

Southern blot analysis was used to establish the integration pattern of soybean event pDAB9582.814.19.1. These experiments generated data which demonstrated the integration and integrity of the cry1Ac and cry1F transgenes within the soybean genome. Soybean event pDAB9582.814.19.1 was characterized as a full length, simple integration event containing a single copy of the cry1Ac and cry1F plant transcription unit (PTU) from plasmid pDAB9582.

Southern blot data suggested that a T-strand fragment inserted into the genome of soybean event pDAB9582.814.19.1. Detailed Southern blot analysis was conducted using probes specific to the cry1Ac and cry1F gene, contained in the T-strand integration region of pDAB9582.814.19.1, and descriptive restriction enzymes that have cleavage sites located within the plasmid and produce hybridizing fragments internal to the plasmid or fragments that span the junction of the plasmid with soybean genomic DNA (border fragments). The molecular weights indicated from the Southern hybridization for the combination of the restriction enzyme and the probe were unique for the event, and established its identification patterns. These analyses also showed that the plasmid fragment had been inserted into soybean genomic DNA without rearrangements of the cry1Ac and cry1F PTU.

#### Example 4.1: Soybean Leaf Sample Collection and Genomic DNA (gDNA) Isolation

Genomic DNA was extracted from leaf tissue harvested from individual soybean plants containing soybean event pDAB9582.814.19.1. In addition, gDNA was isolated from a conventional soybean plant, Maverick, which contains the genetic background that is representative of the substance line, absent the cry1Ac and cry1F genes. Individual genomic DNA was extracted from lyophilized leaf tissue following the standard CTAB method (Sambrook et al (1989)). Following extraction, the DNA was quantified spectrofluorometrically using PICO GREEN reagent (Invitrogen, Carlsbad, Calif.). The DNA was then visualized on an agarose gel to confirm values from the PICO GREEN analysis and to determine the DNA quality.

#### Example 4.2: DNA Digestion and Separation

For Southern blot molecular characterization of soybean event pDAB9582.814.19.1, ten micrograms (10 μg) of genomic DNA was digested. Genomic DNA from the soybean event pDAB9582.814.19.1 and non-transgenic soybean line Maverick was digested by adding approximately five units of selected restriction enzyme per μg of DNA and the corresponding reaction buffer to each DNA sample. Each sample was incubated at approximately 37° C. overnight. The restriction enzymes AseI, HindIII, NsiI, and NdeI were used individually for the single digests (New England Biolabs, Ipswich, Mass.). The restriction enzymes NotI and ApaI were used together for a double digestion (New England Biolabs, Ipswich, Mass.). In addition, a positive hybridization control sample was prepared by combining plasmid DNA, pDAB9582 with genomic DNA from the non-transgenic soybean variety, Maverick. The plasmid



US 9,695,441 B2

29

DNA/genomic DNA cocktail was digested using the same procedures and restriction enzyme as the test samples.

After the digestions were incubated overnight, 25  $\mu$ L QUICK-PRECIP PLUS SOLUTION (Edge Biosystems, Gaithersburg, Md.) was added and the digested DNA samples were precipitated with isopropanol. The precipitated DNA pellet was resuspended in 15  $\mu$ L of 1 $\times$  loading buffer (0.01% bromophenol blue, 10.0 mM EDTA, 10.0% glycerol, 1.0 mM Tris pH 7.5). The DNA samples and molecular size markers were then electrophoresed through 0.85% agarose gels with 0.4 $\times$ TAE buffer (Fisher Scientific, Pittsburgh, Pa.) at 35 volts for approximately 18-22 hours to achieve fragment separation. The gels were stained with ethidium bromide (Invitrogen, Carlsbad, Calif.) and the DNA was visualized under ultraviolet (UV) light.

#### Example 4.3: Southern Transfer and Membrane Treatment

Southern blot analysis was performed essentially as described by Memelink, et al. (1994). Briefly, following electrophoretic separation and visualization of the DNA fragments, the gels were depurinated with 0.25M HCl for approximately 20 minutes, and then exposed to a denaturing solution (0.4 M NaOH, 1.5 M NaCl) for approximately 30 minutes followed by neutralizing solution (1.5 M NaCl, 0.5 M Tris pH 7.5) for at least 30 minutes. Southern transfer was performed overnight onto nylon membranes using a wicking system with 10 $\times$ SSC. After transfer the DNA was bound to the membrane by UV crosslinking following by briefly washing membrane with a 2 $\times$ SSC solution. This process produced Southern blot membranes ready for hybridization.

#### Example 4.4: DNA Probe Labeling and Hybridization

The DNA fragments bound to the nylon membrane were detected using a labeled probe (Table 6). Probes were generated by a PCR-based incorporation of a digoxigenin (DIG) labeled nucleotide, [DIG-11]-dUTP, into the DNA fragment amplified from plasmid pDAB9582 using primers specific to gene elements. Generation of DNA probes by PCR synthesis was carried out using a PCR DIG Probe Synthesis Kit (Roche Diagnostics, Indianapolis, Ind.) following the manufacturer's recommended procedures.

Labeled probes were analyzed by agarose gel electrophoresis to determine their quality and quantity. A desired amount of labeled probe was then used for hybridization to the target DNA on the nylon membranes for detection of the specific fragments using the procedures essentially as described for DIG EASY HYB SOLUTION (Roche Diagnostics, Indianapolis, Ind.). Briefly, nylon membrane blots containing fixed DNA were briefly washed with 2 $\times$ SSC and pre-hybridized with 20-25 mL of pre-warmed DIG EASY HYB SOLUTION in hybridization bottles at approximately 45-55 $^{\circ}$  C. for about 2 hours in a hybridization oven. The pre-hybridization solution was then decanted and replaced with  $\sim$ 15 mL of pre-warmed DIG EASY HYB SOLUTION containing a desired amount of specific probes denatured by boiling in a water bath for approximately five minutes. The hybridization step was then conducted at approximately 45-55 $^{\circ}$  C. overnight in the hybridization oven.

At the end of the probe hybridization, DIG EASY HYB SOLUTIONS containing the probes were decanted into clean tubes and stored at approximately -20 $^{\circ}$  C. These probes could be reused up to two times according to the manufacturer's recommended procedure. The membrane

30

blots were rinsed briefly and washed twice in clean plastic containers with low stringency wash buffer (2 $\times$ SSC, 0.1% SDS) for approximately five minutes at room temperature, followed by washing twice with high stringency wash buffer (0.1 $\times$ SSC, 0.1% SDS) for 15 minutes each at approximately 65 $^{\circ}$  C. The membrane blots briefly washed with 1 $\times$  Maleic acid buffer from the DIG WASH AND BLOCK BUFFER SET (Roche Diagnostics, Indianapolis, Ind.) for approximately 5 minutes. This was followed by blocking in a IX blocking buffer for 2 hours and an incubation with anti-DIG-AP (alkaline phosphatase) antibody (Roche Diagnostics, Indianapolis, Ind.) in 1 $\times$  blocking buffer also for a minimum of 30 minutes. After 2-3 washes with 1 $\times$  washing buffer, specific DNA probes remain bound to the membrane blots and DIG-labeled DNA standards were visualized using CDP-STAR CHEMILUMINESCENT NUCLEIC ACID DETECTION SYSTEM (Roche Diagnostics, Indianapolis, Ind.) following the manufacturer's recommendation. Blots were exposed to chemiluminescent film for one or more time points to detect hybridizing fragments and to visualize molecular size standards. Films were developed with an ALL-PRO 100 PLUS film developer (Konica Minolta, Osaka, Japan) and images were scanned. The number and sizes of detected bands were documented for each probe. DIG-LABELED DNA MOLECULAR WEIGHT MARKER II (DIG MWM II) and DIG-LABELED DNA MOLECULAR WEIGHT MARKER VII (DIG MWM VII), visible after DIG detection as described, were used to determine hybridizing fragment size on the Southern blots.

TABLE 6

Location and length of probes used in Southern analysis.		
Probe Name	Genetic Element	Length (bp)
Cry1Ac	cry1Ac	1720
Cry1F	cry1F	1746
specR	Spectinomycin resistance gene	750
OriRep	Ori Rep	852
trfA	Replication initiation protein trfA	1119

#### Example 4.5: Southern Blot Results

Expected and observed fragment sizes with a particular digest and probe, based on the known restriction enzyme sites of the cry1Ac and cry1F PTU, are given in Table 7. Two types of fragments were identified from these digests and hybridizations: internal fragments where known enzyme sites flank the probe region and are completely contained within the insertion region of the cry1Ac and cry1F PTU, and border fragments where a known enzyme site is located at one end of the probe region and a second site is expected in the soybean genome. Border fragment sizes vary by event because, in most cases, DNA fragment integration sites are unique for each event. The border fragments provide a means to locate a restriction enzyme site relative to the integrated DNA and to evaluate the number of DNA insertions. Southern blot analyses completed on multiple generations of soybean containing soybean event pDAB9582.814.19.1 produced data which suggested that a low copy, intact cry1Ac and cry1F PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.814.19.1.

US 9,695,441 B2

31

TABLE 7

Predicted and observed hybridizing fragments in Southern blot analysis.  
 1. Expected fragment sizes are based on the plasmid map of pDAB9582.  
 2. Observed fragment sizes are considered approximately from these analyses and are based on the indicated sizes of the DIG-LABELED DNA MOLECULAR WEIGHT MARKER II and MARK VII fragments.

DNA Probe	Restriction Enzymes	Samples	Expected Fragment Sizes (bp) <sup>1</sup>	Observed Fragment Size (bp) <sup>2</sup>
Cry1Ac	AseI	pDAB9582	13476	>14000
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	>7286	~7400
	Nsi I	pDAB9582	15326	>15000
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	>9479	>10000
	Not I + ApaLI	pDAB9582	4550	~4500
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	4550	~4500
	NdeI	pDAB9582	8071	~8000
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	5569	~7500
		pDAB9582	11044	11000
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	>9479	>10000
Cry1F	Hind III	pDAB9582	7732	~7700
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	7732	~7700
	NsiI	pDAB9582	15320	~15000
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	none	none
SpecR	NsiI	pDAB9582	15320	~15000
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	none	none
trfA	NsiI	pDAB9582	15320	~15000
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	none	none
oriREP	NdeI	pDAB9582	5239	~5000
		Maverick	none	none
		Soybean Event pDAB9582.814.19.1	none	none

The restriction enzymes AseI and NsiI bind and cleave unique restriction sites in plasmid pDAB9582. Subsequently, these enzymes were selected to characterize the cry1Ac gene insert in soybean event pDAB9582.814.19.1. Border fragments of >7286 bp or >9479 bp were predicted to hybridize with the probe following AseI and NsiI digests, respectively (Table 7). Single cry1Ac hybridization bands of about 7400 and >10000 bp were observed when AseI and NsiI digests were used, respectively. The hybridization of the probe to bands of this size suggests the presence of a single site of insertion for the cry1Ac gene in the soybean genome of soybean event pDAB9582.814.19.1. Restriction enzymes NotI and ApaLI were selected to perform a double digestion and to release a fragment which contains the cry1Ac plant transcription unit (PTU; promoter/gene/terminator) (Table 7). The predicted 4550 bp fragments were observed with the probe following NotI and ApaLI double digestion. Results obtained with the enzyme digestion of the pDAB9582.814.19.1 samples followed by probe hybridization indicated that an intact cry1Ac PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.814.19.1.

The restriction enzymes NdeI and NsiI bind and cleave restriction sites in plasmid pDAB9582. Subsequently, these enzymes were selected to characterize the cry1F gene insert in soybean event pDAB9582.814.19.1. Border fragments of

32

>5569 bp and >9479 were predicted to hybridize with the probe following the NdeI and NsiI digests, respectively (Table 7). Single cry1F hybridization bands of ~7500 bp and >10000 bp were observed when NdeI and NsiI were used, respectively. The hybridization of the probe to bands of this size suggests the presence of a single site of insertion for the cry1F gene in the soybean genome of soybean event pDAB9582.814.19.1. Restriction enzyme, HindIII, was selected to release a fragment which contains the cry1F plant transcription unit (PTU; promoter/gene/terminator) (Table 7). The predicted 7732 bp fragment was observed with the probe following the HindIII digestions. Results obtained with the enzyme digestion of the pDAB9582.814.19.1 samples followed by probe hybridization indicated that an intact cry1F PTU from plasmid pDAB9582 was inserted into the soybean genome of soybean event pDAB9582.814.19.1.

#### Example 4.6: Absence of Backbone Sequences

Southern blot analysis was also conducted to verify the absence of the spectinomycin resistance gene (specR), Ori Rep element and replication initiation protein trfA (trf A element) in soybean event pDAB9582.814.19.1. No specific hybridization to spectinomycin resistance, Ori Rep element or trf A element is expected when appropriate positive (pDAB9582 added to Maverick genomic DNA) and negative (Maverick genomic DNA) controls are included for Southern analysis. Following the NsiI digestion and hybridization with the specR specific probe, one expected size band of 15320 bp was observed in the positive control sample (pDAB9582 added to Maverick genomic DNA). The specR probe did not hybridize to samples of the negative control and soybean event pDAB9582.814.19.1. Similarly, one expected size band of 15320 bp was detected in the positive control sample (pDAB9582 plus maverick) but absent from the samples of the negative control and soybean event pDAB9582.814.19.1 after NsiI digestion and hybridization with trfA probe. Another expected size band of 5329 bp was detected in the positive control sample (pDAB9582 added to Maverick genomic DNA) but absent from the samples of the negative control and soybean event pDAB9582.814.19.1 after NdeI digestion and hybridization with OriRep specific probe. These data indicate the absence of spectinomycin resistance gene, Ori Rep element and replication initiation protein trfA in soybean event pDAB9582.814.19.1.

#### Example 5: Agronomic and Yield Field Trial and Herbicide Tolerance

To test the agronomic characteristics and efficacy of soybean event pDAB9582.814.19.1 the event was planted in an efficacy trial at Santa Isabel, Puerto Rico in October 2010 and February 2011. The cultivar Maverick, which was originally transformed to produce event pDAB9582.814.19.1, was planted in each nursery and included as a control in the experiments. Seed for the T3 nursery was derived from single plant selections at the T2 stage and seed for the T4 nursery was derived from single plant selections at the T3 stage. Four lineages of the event were tested each generation. Each lineage was planted in a plot which was 4 rows wide and 7.5 feet long. The spacing between rows was 30 inches. Plots were grown under lights for approximately 2.5 weeks to compensate for the short day length in Puerto Rico. Each nursery was sprayed with glufosinate at a rate of 411 g ae/ha. One plot of the control

US 9,695,441 B2

33

plants, Maverick, was sprayed with the same rate of glufosinate and a second plot was non-sprayed and used as control comparison for the event.

Data was collected on emergence, general appearance, vigor, height, lodging, and maturity. Herbicide tolerance was assessed by visually looking for chlorosis, leaf necrosis and plant death (Table 8).

For comparisons of soybean event pDAB9582.814.19.1 with Maverick, only data from the unsprayed block of Maverick were used. For comparison of the sprayed and non-sprayed treatments, data from the soybean event pDAB9582.814.19.1 block sprayed with a given treatment were compared with data from the Maverick control non-sprayed block. Soybean event pDAB9582.814.19.1 showed tolerance to the glufosinate herbicide application. In contrast, none of the Maverick plants were tolerant to the herbicide treatments.

TABLE 8

Comparison of soybean event pDAB9582.814.19.1 to Maverick. Values are averages from T <sub>3</sub> and T <sub>4</sub> nurseries. Each nursery of soybean event pDAB9582.814.19.1 was sprayed with glufosinate at the V3 stage at a rate of 411 g ae/ha.						
Event	Emergence (%)	Appearance (1 = poor to 9 = good)	Vigor (1 = poor to 9 = good)	Height (cm)	Lodging (%)	Maturity (day)
pDAB9582.814.19.1	90	8	8	69	1	91
Maverick	82	8	8	64	1	91

30

#### Example 6: Characterization of Insecticidal Activity for Soybean Event 9582.814.19.1

Field and greenhouse evaluations were conducted to characterize the activity of Cry1Ac and Cry1F in soybean event pDAB9582.814.19.1 against lab reared soybean pests including *Anticarsia gemmatilis* (velvetbean caterpillar), *Pseudoplusia includens* (soybean looper) and *Spodoptera frugiperda* (fall armyworm). Soybean event pDAB9582.814.19.1 was compared against non-transformed soybean variety Maverick, to determine the level of plant protection provided by the Cry1F and Cry1Ac proteins.

Greenhouse trials were conducted on approximately four week old plants. Fifteen plants were used to evaluate the soybean event pDAB9582.814.19.1 and the Maverick control. For each insect species tested (*Anticarsia gemmatilis*, *Pseudoplusia includens*, and *Spodoptera frugiperda*) 3 leaf punches were made from each plant for a total of 45 leaf discs/plant/insect species. The 1.4 cm diameter (or 1.54 cm<sup>2</sup>) leaf punches were placed in a test arena on top of 2% water agar, infested with one neonate larvae and sealed with a perforated plastic lid. Mortality and leaf consumption were rated 4 days after infestation. Larvae that were not responsive to gentle probing were considered dead. Leaf damage was assessed by visually scoring the percentage of leaf punch consumed by the insect.

Field evaluations were conducted by collecting leaf samples from seed increase nursery plots in Santa Isabel, Puerto Rico and sending these leaves to Indianapolis, Ind. for testing. The nursery plot for soybean event pDAB9582.814.19.1 was planted in February 2011 and consisted of approximately 180 plants arranged in four rows. Each row was 2.3 m long and spaced 76.2 cm apart; individual plants were spaced 5.1 cm apart within each row. In March 2011, one fully-expanded, mainstem trifoliate leaf,

34

located approximately four nodes below the meristem, was excised from 10 soybean event pDAB9582.814.19.1 plants and 10 'Maverick' plants. The leaves were placed in labeled plastic bags, (one per bag) and sealed. The bagged leaves were packed and transferred to the laboratory. In the laboratory, one or two 3.33 cm (1.31 in) diameter leaf discs were punched from each trifoliate leaf to provide a total of 16 leaf discs. Each leaf disc was placed in a test arena on top of 2% agar, infested with one neonate *S. frugiperda* larva, and sealed with a perforated plastic lid. The leaf discs were held in a controlled environment chamber for 7 days, at which time mortality and leaf consumption were rated. Larvae not responsive to gentle probing were considered dead. Leaf damage was assessed by visually scoring the percentage of leaf punch consumed by the insect.

The results obtained from these replicated experiments indicated the soybean event pDAB9582.814.19.1 sustained

significantly lower damage than the Maverick control plants for all insects tested. Thus, the soybean event pDAB9582.814.19.1 has insecticidal activity over this broad host range.

#### Example 7: Sequence of Soybean Event pDAB9582.814.19.1

SEQ ID NO:14 provides the sequence of soybean event pDAB9582.814.19.1. This sequence contains the 5' genomic flanking sequence, the T-strand insert of pDAB9582 and 3' genomic flanking sequences. With respect to SEQ ID NO:14, residues 1-1400 are 5' genomic flanking sequence, residues 1401-1536 are residues of a rearrangement from the pDAB9582 plasmid and 1537-13896 are residues of the pDAB9582 T-strand insert, and residues 13897-15294 are 3' flanking sequence. The junction sequence or transition with respect to the 5' end of the insert thus occurs at residues 1400-1401 of SEQ ID NO:14. The junction sequence or transition with respect to the 3' end of the insert thus occurs at residues 13896-13897 of SEQ ID NO:14.

It should be noted that progeny from soybean event pDAB9582.814.19.1 may have sequences which slightly deviate from SEQ ID NO:14. During the introgression and breeding process of introducing soybean event pDAB9582.814.19.1 into the genome of plant cells, it is not uncommon for some deletions or other alterations of the insert to occur. Moreover, errors in PCR amplification can occur which might result in minor sequencing errors. For example, flanking sequences listed herein were determined by generating amplicons from soybean genomic DNAs, and then cloning and sequencing the amplicons. It is not unusual to find slight differences and minor discrepancies in sequences generated and determined in this manner, given the many rounds of amplification that are necessary to generate enough amplicon for sequencing from genomic

US 9,695,441 B2

35

DNAs. One skilled in the art should recognize and be put on notice that any adjustments needed due to these types of common sequencing errors or discrepancies are within the scope of the subject invention. Thus, the relevant segment of the plasmid sequence provided herein might comprise some minor variations. Thus, a plant comprising a polynucleotide having some range of identity with the subject insert sequence is within the scope of the subject invention. Identity to the sequence of SEQ ID NO:14 can be a polynucleotide sequence having at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity with a sequence exemplified or described herein. Thus, some differences between SEQ ID NO:14 and soy-

36

bean event pDAB9582.814.19.1 progeny plants may be identified and are within scope of the present invention.

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 14

<210> SEQ ID NO 1

<211> LENGTH: 1836

<212> TYPE: DNA

<213> ORGANISM: Glycine max

<400> SEQUENCE: 1

ttaacaatga ccaagattta tgctatatag aagacttgga gggcttaagg ctatgatata	60
ttatggatga tatggttctg atttgtgtag ttctgaagga tcaaatcaac catttgttgg	120
tacaatggga agaaaaaatg ttttcatcat tccactctat tgaaaaagat ccaacaattg	180
taacaccccg acgaatcaca ccggaagag aagaatccaa agatttgtta ggtatgagac	240
tgtatagttg atgaaaactt aaaaaaatta attggtacta cttataccaa caagatgcat	300
atatttttcg atagcctatc acataagaac ttcatagtta agggtgctta acttggagta	360
gttatgaaat gagtgacctt taaaaataat tattgtctta ggttattgta tgaaaataaa	420
aaataataat aaatatacat aaaaaataat aattttataa aattaacctt atattatcat	480
taattttattt ttagattttg ttattcatta ttaatatatg aggtataaat gaaaaatata	540
attaatgtca cattaaaaaa ttaaaatgat aattattttg aaacaaatta tttattttta	600
tacgacaatt ataatagaaa tttagagta aaaaaaatt gaaaattcat aaaatatatg	660
aatatattca tttctcctat ccgtcaaata aatctgctcc ataatttate taagcattgg	720
tctttagtgg cagagtaata aaatttttagc aattattagt tagtacagat acatttaaag	780
aaataatata ttttagcaac tagaagtta taaaaagttt taaattataa agacttatat	840
ataaatttag taaaactaga tggatgtccc aagtaatttt tatataacta ttctcgtaca	900
acattaatga aaatccttgt tctattattt atatgtatat tattatttta ttttgaaca	960
atatgggatt aaaaactcct ataaaataaa tcttagaata agttttccta acatgttttt	1020
tttatggatg ttttcctaac atgtttggtt atcttagttt tgctttaatt ttgtcggatt	1080
atttttggac tttattaggt aattttgata aaacttttag ttgatgttag tagtttactc	1140
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tacaagagtt cgccaatcaa gaggatttga agagagtaaa atattatgag aagtcacatg	1260
tgaagaaaat ccaaccattg gaataaaaaa taaagttttt tctttggaat tgctaagtct	1320
acagcactta ttggtaactg tcttaaaaaa gaaactctag ctatatattg caattgatat	1380
tcataaatca aacttctcta tgaaataacc gcggtgcgca tcggtgctg ttgatccgc	1440
gcaagttggg atcttgaagc aagttccgct catcactaag tcgcttagca tgtttgacct	1500
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US 9,695,441 B2

37

38

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taggccccgaa tagtttgaaa ttagaaaagct cgcaattgag gtctacaggc caaatcgcgt	1620
cttagccgta caatattact caccggatcc taaccgggtg gatcatgggc cgcgattaaa	1680
aatctcaatt atatttggtc taatttagtt tggattgag taaaacaaat tcggcgccat	1740
gccccgggcaa gggcgccgac aagtttgtag aaaaaagcag gctccgcggt gactgactga	1800
aaagcttgct gacctgcagg tcaacggatc aggata	1836

&lt;210&gt; SEQ ID NO 2

&lt;211&gt; LENGTH: 1550

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Glycine max

&lt;400&gt; SEQUENCE: 2

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agtacattaa aaacgtccgc aatatgatat tcattaattt tatattatct aaaagagtta	180
aaagagaaaa aagaatatg acaatttttt tctttcacat cttctaacct aaaagtatga	240
ctctatggag gctaagttaa gaaaaagata cggatctagg gtgtggaac atcaatggtc	300
aaactccttt atatttcaat caattgggtt ttgctttatc tttacatttt ctccttttat	360
tttccacgtc tattcaaac tacttgtag cgggtgatta ctcttttttc ttttatagat	420
gccattatt tctctcctat gtattaaatt agagtatatt gtcttgaag tgacttagta	480
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&lt;220&gt; FEATURE:

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US 9,695,441 B2

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US 9,695,441 B2

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US 9,695,441 B2

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US 9,695,441 B2

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US 9,695,441 B2

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US 9,695,441 B2

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US 9,695,441 B2

51

52

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US 9,695,441 B2

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US 9,695,441 B2

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US 9,695,441 B2

57

58

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US 9,695,441 B2

59

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US 9,695,441 B2

61

62

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ctgctggaat	gtgaaagggc	acgtagatgt	agaagaacag	aacaatcacc	gctctgtcct	10680
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US 9,695,441 B2

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ttgtgaaaaac aagaaaaaat ttggtgtaag ctattttctt tgaagtactg aggatacaac 12300  
ttcagagaaa tttgtaagtt tgtagatctc catgtctccg gagaggagac cagttgagat 12360  
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gtctaagggt cagagggtt agcgggatga agcaaaagt tccgattgta acaagatatg 13200  
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taatacatag cggccgggtt tctagtacc ggttaggac cgtttaaact cgaggctagc 13740  
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gttaaaagag aaaaaagaaa tatgacaatt tttttcttc acatcttcta acctaaaagt 13980  
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ggtcaactcc ttttatattt caatcaattg ggttttgcct tatctttaca tttctcctt 14100  
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tatattttaa gataaaactt actttcctgc aataaaataa agaaaaggac agtcatacaa 14460



## US 9,695,441 B2

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agtttttaga	cagaaaagga	aagtaaatta	tagagataat	gaagtttgct	cttttaaatt	14940
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actaaagctt	caattaatta	tatccaataa	acggtattgg	tgtatgatgt	tatgatagca	15180
aatagataat	ctaactata	cgagccacaa	aaggggcatg	aactctatct	cgaagaaatt	15240
ggagatgaag	ggattgagat	tggcaccttg	tgctattatt	gccactaat	catt	15294

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The invention claimed is:

1. A polynucleotide comprising SEQ ID NO:14.
2. A soybean plant, seed, or other part of said plant comprising the polynucleotide of claim 1.
3. An isolated polynucleotide that is diagnostic for soybean event 9582.814.19.1, wherein said polynucleotide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:14.

4. An isolated polynucleotide in claim 3 comprising one or more sequences selected from the group consisting of nucleotides 1385-1415 of SEQ ID NO:1, nucleotides 1350-1450 of SEQ ID NO:1, nucleotides 1300-1500 of SEQ ID NO:1, nucleotides 1200-1600 of SEQ ID NO:1, nucleotides 137-168 of SEQ ID NO:2, nucleotides 103-203 of SEQ ID NO:2, and nucleotides 3-303 of SEQ ID NO:2.

\* \* \* \* \*

# **Exhibit G**

(12) **United States Patent**  
**Barbour et al.**(10) **Patent No.:** **US 8,901,378 B2**(45) **Date of Patent:** **\*Dec. 2, 2014**(54) **CORN EVENT TC1507 AND METHODS FOR DETECTION THEREOF**

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(73) Assignee: **Pioneer Hi-Bred International, Inc.**, Johnston, IA (US)

(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 1071 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **12/914,271**

(22) Filed: **Oct. 28, 2010**

(65) **Prior Publication Data**

US 2011/0099662 A1 Apr. 28, 2011

**Related U.S. Application Data**

(60) Division of application No. 12/333,044, filed on Mar. 13, 2009, now Pat. No. 7,989,607, which is a continuation of application No. 11/774,236, filed on Jul. 6, 2007, now Pat. No. 7,514,544, which is a division of application No. 10/837,105, filed on Apr. 30, 2004, now Pat. No. 7,288,643.

(60) Provisional application No. 60/467,772, filed on May 2, 2003.

(51) **Int. Cl.**

**A01H 1/00** (2006.01)

**C12N 15/82** (2006.01)

**C12Q 1/68** (2006.01)

(52) **U.S. Cl.**

CPC ..... **C12N 15/8277** (2013.01); **C12Q 1/6895**

(2013.01); **C12Q 1/686** (2013.01); **C12N 15/8286** (2013.01); **Y10S 435/975** (2013.01)  
 USPC ..... **800/302**; 800/265; 435/975

(58) **Field of Classification Search**

None

See application file for complete search history.

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Primary Examiner — David H Kruse

(74) Attorney, Agent, or Firm — Ballard Spahr LLP

(57) **ABSTRACT**

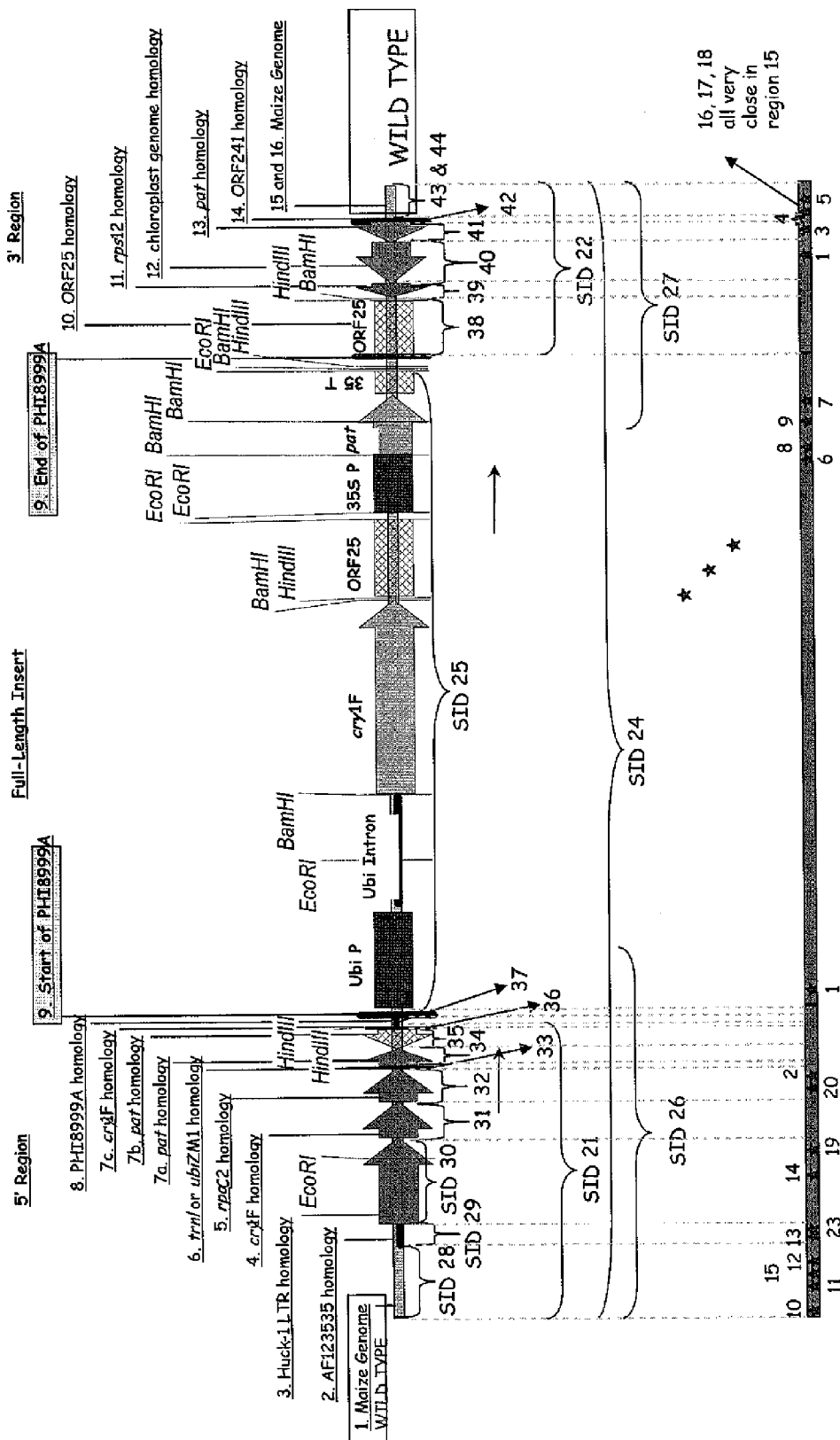
The invention provides DNA compositions that relate to transgenic insect resistant maize plants. Also provided are assays for detecting the presence of the maize TC1507 event based on the DNA sequence of the recombinant construct inserted into the maize genome and the DNA sequences flanking the insertion site. Kits and conditions useful in conducting the assays are provided.

**8 Claims, 1 Drawing Sheet**

## U.S. Patent

Dec. 2, 2014

US 8,901,378 B2



This bottom diagram shows where primers are located by SID number. The green is flanking, the grey is insert. The red stars are rough (not to scale) locations of primers and the number is the SID.

US 8,901,378 B2

1

**CORN EVENT TC1507 AND METHODS FOR  
DETECTION THEREOF****CROSS-REFERENCE TO RELATED  
APPLICATIONS**

This application is a divisional of U.S. Ser. No. 12/333,044, filed Mar. 13, 2007, which is a continuation of U.S. Ser. No. 11/774,236 filed Jul. 6, 2007, which is a divisional of 10/837,105, filed Apr. 30, 2004, which claims the benefit of U.S. Provisional Application Ser. No. 60/467,772, filed May 2, 2003, the contents of all of which are herein incorporated by reference in their entirety.

**FIELD OF THE INVENTION**

The present invention relates to the field of plant molecular biology, specifically the invention relates to a DNA construct for conferring insect resistance to a plant. The invention more specifically relates to an insect resistant corn plant TC1507 and to assays for detecting the presence of corn plant TC1507 DNA in a sample and compositions thereof.

**BACKGROUND OF THE INVENTION**

This invention relates to the insect resistant corn (*Zea mays*) plant TC1507, also referred to as maize line TC1507 or maize event TC1507, and to the DNA plant expression construct of corn plant TC1507 and the detection of the transgene/flanking insertion region in corn plant TC1507 and progeny thereof.

Corn is an important crop and is a primary food source in many areas of the world. Damage caused by insect pests is a major factor in the loss of the world's corn crops, despite the use of protective measures such as chemical pesticides. In view of this, insect resistance has been genetically engineered into crops such as corn in order to control insect damage and to reduce the need for traditional chemical pesticides. One group of genes which have been utilized for the production of transgenic insect resistant crops are the delta-endotoxins from *Bacillus thuringiensis* (B.t.). Delta-endotoxins have been successfully expressed in crop plants such as cotton, potatoes, rice, sunflower, as well as corn, and have proven to provide excellent control over insect pests. (Perlak, F. J et al. (1990) *Bio/Technology* 8, 939-943; Perlak, F. J. et al. (1993) *Plant Mol. Biol.* 22: 313-321; Fujimoto H. et al. (1993) *Bio/Technology* 11: 1151-1155; Tu et al. (2000) *Nature Biotechnology* 18:1101-1104; PCT publication number WO 01/13731; and Bing J W et al. (2000) Efficacy of Cry1F Transgenic Maize, 14<sup>th</sup> Biennial International Plant Resistance to Insects Workshop, Fort Collins, Colo.).

The expression of foreign genes in plants is known to be influenced by their location in the plant genome, perhaps due to chromatin structure (e.g., heterochromatin) or the proximity of transcriptional regulatory elements (e.g., enhancers) close to the integration site (Weising et al., *Ann. Rev. Genet* 22:421-477, 1988). At the same time the presence of the transgene at different locations in the genome will influence the overall phenotype of the plant in different ways. For this reason, it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of an introduced gene of interest. For example, it has been observed in plants and in other organisms that there may be a wide variation in levels of expression of an introduced gene among events. There may also be differences in spatial or temporal patterns of expression, for example, differences in the relative expression of a transgene in various plant

2

tissues, that may not correspond to the patterns expected from transcriptional regulatory elements present in the introduced gene construct. For this reason, it is common to produce hundreds to thousands of different events and screen those events for a single event that has desired transgene expression levels and patterns for commercial purposes. An event that has desired levels or patterns of transgene expression is useful for introgressing the transgene into other genetic backgrounds by sexual outcrossing using conventional breeding methods. Progeny of such crosses maintain the transgene expression characteristics of the original transformant. This strategy is used to ensure reliable gene expression in a number of varieties that are well adapted to local growing conditions.

It would be advantageous to be able to detect the presence of a particular event in order to determine whether progeny of a sexual cross contain a transgene of interest. In addition, a method for detecting a particular event would be helpful for complying with regulations requiring the pre-market approval and labeling of foods derived from recombinant crop plants, for example, or for use in environmental monitoring, monitoring traits in crops in the field, or monitoring products derived from a crop harvest, as well as for use in ensuring compliance of parties subject to regulatory or contractual terms.

It is possible to detect the presence of a transgene by any nucleic acid detection method known in the art including, but not limited to, the polymerase chain reaction (PCR) or DNA hybridization using nucleic acid probes. These detection methods generally focus on frequently used genetic elements, such as promoters, terminators, marker genes, etc., because for many DNA constructs, the coding region is interchangeable. As a result, such methods may not be useful for discriminating between different events, particularly those produced using the same DNA construct or very similar constructs unless the DNA sequence of the flanking DNA adjacent to the inserted heterologous DNA is known. For example, an event-specific PCR assay is described in U.S. Pat. No. 6,395,485 for the detection of elite event GAT-ZM1. Accordingly, it would be desirable to have a simple and discriminative method for the identification of event TC1507.

**SUMMARY OF THE INVENTION**

This invention relates preferably to methods for producing and selecting an insect resistant monocot crop plant. More specifically, a DNA construct is provided that when expressed in plant cells and plants confers resistance to insects. According to one aspect of the invention, a DNA construct, capable of introduction into and replication in a host cell, is provided that when expressed in plant cells and plants confers insect resistance to the plant cells and plants. The DNA construct is comprised of a DNA molecule named PHI8999A and it includes two transgene expression cassettes. The first expression cassette comprises a DNA molecule which includes the promoter, 5' untranslated exon, and first intron of the maize ubiquitin (Ubi-1) gene (Christensen et al. (1992) *Plant Mol. Biol.* 18:675-689 and Christensen and Quail (1996) *Transgenic Res.* 5:213-218) operably connected to a DNA molecule encoding a B.t.  $\delta$ -endotoxin identified as Cry1F (U.S. Pat. Nos. 5,188,960 and 6,218,188) operably connected to a DNA molecule comprising a 3' ORF25 transcriptional terminator isolated from *Agrobacterium tumefaciens* (Barker et al. (1983) *Plant Mol. Biol.* 2:335-350). The second transgene expression cassette of the DNA construct comprises a DNA molecule of the cauliflower mosaic virus (CaMV) 35S promoter (Odell J. T. et al. (1985) *Nature* 313: 810-812; Mitsu-hara et al. (1996) *Plant Cell Physiol.* 37: 49-59) operably

## US 8,901,378 B2

3

connected to a DNA molecule encoding a phosphinothricin acetyltransferase (PAT) gene (Wohleben W. et al. (1988) *Gene* 70: 25-37) operably connected to a DNA molecule comprising a 3' transcriptional terminator from (CaMV) 35S (see Mitsuhashi et al. (1996) *Plant Cell Physiol.* 37: 49-59). Plants containing the DNA construct are also provided.

According to another aspect of the invention, compositions and methods are provided for identifying a novel corn plant designated TC1507, which methods are based on primers or probes which specifically recognize the 5' and/or 3' flanking sequence of TC1507. DNA molecules are provided that comprise primer sequences that when utilized in a PCR reaction will produce amplicons unique to the transgenic event TC1507. These molecules may be selected from the group consisting of:

(SEQ ID NO: 1)  
5' -GTAGTACTATAGATTATATTATTCGTAGAG-3' ;

(SEQ ID NO: 2)  
5' -GCCATACAGAACTCAAAATCTTTTCCGGAG-3' ;

(SEQ ID NO: 23)  
5' -CTTCAACAAGTGTGACAAA-3' ;

(SEQ ID NO: 3)  
5' -TGTGGTGTGGTCTGTCTCTAA-3' ;

(SEQ ID NO: 4)  
5' -AGCACCTTTTCATTCTTTTCATATAC-3' ;

(SEQ ID NO: 5)  
5' -GACCTCCCCA CAGGCATGAT TGATC-3' ;

and complements thereof. The corn plant and seed comprising these molecules is an aspect of this invention. Further, kits utilizing these primer sequences for the identification of the TC1507 event are provided.

An additional aspect of the invention relates to the specific flanking sequences of TC1507 described herein, which can be used to develop specific identification methods for TC1507 in biological samples. More particularly, the invention relates to the 5' and/or 3' flanking regions of TC1507, SEQ ID NO:21 and SEQ ID NO:22, respectively, which can be used for the development of specific primers and probes. The invention further relates to identification methods for the presence of TC1507 in biological samples based on the use of such specific primers or probes.

According to another aspect of the invention, methods of detecting the presence of DNA corresponding to the corn event TC1507 in a sample are provided. Such methods comprise: (a) contacting the sample comprising DNA with a DNA primer set, that when used in a nucleic acid amplification reaction with genomic DNA extracted from corn event TC1507 produces an amplicon that is diagnostic for corn event TC1507; (b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and (c) detecting the amplicon.

DNA molecules that comprise the novel transgene/flanking insertion region, SEQ ID NO: 26 and SEQ ID NO: 27 and are homologous or complementary to SEQ ID NO: 26 and SEQ ID NO: 27 are an aspect of this invention.

DNA sequences that comprise the novel transgene/flanking insertion region, SEQ ID NO:26 are an aspect of this invention. DNA sequences that comprise a sufficient length of polynucleotides of transgene insert sequence and a sufficient length of polynucleotides of maize genomic and/or flanking sequence from maize plant TC1507 of SEQ ID NO:26 that are

4

useful as primer sequences for the production of an amplicon product diagnostic for maize plant TC1507 are included.

In addition, DNA sequences that comprise the novel transgene/flanking insertion region, SEQ ID NO:27 are provided. DNA sequences that comprise a sufficient length of polynucleotides of transgene insert sequence and a sufficient length of polynucleotides of maize genomic and/or flanking sequence from maize plant TC1507 of SEQ ID NO:27 that are useful as primer sequences for the production of an amplicon product diagnostic for maize plant TC1507 are included.

According to another aspect of the invention, the DNA sequences that comprise at least 11 or more nucleotides of the transgene portion of the DNA sequence of SEQ ID NO:26 or complements thereof, and a similar length of 5' flanking maize DNA sequence of SEQ ID NO:26 or complements thereof are useful as DNA primers in DNA amplification methods. The amplicons produced using these primers are diagnostic for maize event TC1507. Therefore, the invention also includes the amplicons produced by DNA primers homologous or complementary to SEQ ID NO:26.

According to another aspect of the invention, the DNA sequences that comprise at least 11 or more nucleotides of the transgene portion of the DNA sequence of SEQ ID NO:27 or complements thereof, and a similar length of 3' flanking maize DNA sequence of SEQ ID NO:27 or complements thereof are useful as DNA primers in DNA amplification methods. The amplicons produced using these primers are diagnostic for maize event TC1507. Therefore, the invention also includes the amplicons produced by DNA primers homologous or complementary to SEQ ID NO:27.

More specifically, a pair of DNA molecules comprising a DNA primer set, wherein the DNA molecules are identified as SEQ ID NO: 1 or complements thereof and SEQ ID NO: 2 or complements thereof; SEQ ID NO: 2 or complements thereof and SEQ ID NO: 23 or complements thereof; SEQ ID NO: 3 or complements thereof and SEQ ID NO: 5 or complements thereof; SEQ ID NO: 4 or complements thereof and SEQ ID NO: 5 or complements thereof are aspects of the invention.

Further aspects of the invention include the amplicon comprising the DNA molecules of SEQ ID NO: 1 and SEQ ID NO: 2; the amplicon comprising the DNA molecules of SEQ ID NO: 2 and SEQ ID NO: 23; the amplicon comprising the DNA molecules of SEQ ID NO: 3 and SEQ ID NO: 5; and the amplicon comprising the DNA molecules of SEQ ID NO: 4 and SEQ ID NO: 5.

According to another aspect of the invention, methods of detecting the presence of a DNA molecule corresponding to the TC1507 event in a sample, such methods comprising: (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe, molecule that hybridizes under stringent hybridization conditions with DNA extracted from corn event TC1507 and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA. More specifically, a method for detecting the presence of a DNA molecule corresponding to the TC1507 event in a sample, such methods, consisting of (a) contacting the sample comprising DNA extracted from a corn plant with a DNA probe molecule that consists of sequences that are unique to the event, e.g. junction sequences, wherein said DNA probe molecule hybridizes under stringent hybridization conditions with DNA extracted from corn event TC1507 and does not hybridize under the stringent hybridization conditions with a control corn plant DNA; (b) subjecting the sample and probe to stringent hybridization conditions; and (c) detecting hybridization of the probe to the DNA.



US 8,901,378 B2

5

In addition, a kit and methods for identifying event TC1507 in a biological sample which detects a TC1507 specific region within SEQ ID NO: 24 are provided.

DNA molecules are provided that comprise at least one junction sequence of TC1507 selected from the group consisting of SEQ ID NO: 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56 and 57 and complements thereof; wherein a junction sequence spans the junction between heterologous DNA inserted into the genome and the DNA from the corn cell flanking the insertion site, i.e. flanking DNA, and is diagnostic for the TC1507 event.

According to another aspect of the invention, methods of producing an insect resistant corn plant that comprise the steps of: (a) sexually crossing a first parental corn line comprising the expression cassettes of the present invention, which confers resistance to insects, and a second parental corn line that lacks insect resistance, thereby producing a plurality of progeny plants; and (b) selecting a progeny plant that is insect resistant. Such methods may optionally comprise the further step of back-crossing the progeny plant to the second parental corn line to producing a true-breeding corn plant that is insect resistant.

The present invention provides a method of producing a corn plant that is resistant to insects comprising transforming a corn cell with the DNA construct PHI8999A (SEQ ID NO: 25), growing the transformed corn cell into a corn plant, selecting the corn plant that shows resistance to insects, and further growing the corn plant into a fertile corn plant. The fertile corn plant can be self pollinated or crossed with compatible corn varieties to produce insect resistant progeny.

The invention further relates to a DNA detection kit for identifying maize event TC1507 in biological samples. Preferably the kit of the invention comprises a first primer which specifically recognizes the 5' or 3' flanking region of TC1507, and a second primer which specifically recognizes a sequence within the foreign DNA of TC1507, or within the flanking DNA, for use in a PCR identification protocol. The invention also relates to a kit for identifying event TC1507 in biological samples, which kit comprises a specific probe having a sequence which corresponds or is complementary to, a sequence having between 80% and 100% sequence identity with a specific region of event TC1507. Preferably the sequence of the probe corresponds to a specific region comprising part of the 5' or 3' flanking region of event TC1507.

The methods and kits encompassed by the present invention can be used for different purposes such as, but not limited to the following: to identify event TC1507 in plants, plant material or in products such as, but not limited to, food or feed products (fresh or processed) comprising, or derived from plant material; additionally or alternatively, the methods and kits of the present invention can be used to identify transgenic plant material for purposes of segregation between transgenic and non-transgenic material; additionally or alternatively, the methods and kits of the present invention can be used to determine the quality of plant material comprising maize event TC1507. The kits may also contain the reagents and materials necessary for the performance of the detection method.

This invention further relates to the TC1507 corn plant or its parts, including, but not limited to, pollen, ovules, vegetative cells, the nuclei of pollen cells, and the nuclei of egg cells of the corn plant TC1507 and the progeny derived thereof. The corn plant and seed TC1507 from which the DNA primer molecules of the present invention provide a specific amplification product is an aspect of the invention.

6

The foregoing and other aspects of the invention will become more apparent from the following detailed description and accompanying drawing.

#### BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Linear map showing the transgenic insert PHI8999A, as well as the sequences flanking the transgenic insert. Note: FIG. 1 is not necessarily to scale.

#### DETAILED DESCRIPTION

The following definitions and methods are provided to better define the present invention and to guide those of ordinary skill in the art in the practice of the present invention. Unless otherwise noted, terms are to be understood according to conventional usage by those of ordinary skill in the relevant art. Definitions of common terms in molecular biology may also be found in Rieger et al., *Glossary of Genetics: Classical and Molecular*, 5<sup>th</sup> edition, Springer-Verlag; New York, 1991; and Lewin, *Genes V*, Oxford University Press: New York, 1994. The nomenclature for DNA bases as set forth at 37 CFR 1.822 is used.

As used herein, the term "comprising" means "including but not limited to".

As used herein, the term "corn" means *Zea mays* or maize and includes all plant varieties that can be bred with corn, including wild maize species.

As used herein, the term "TC1507 specific" refers to a nucleotide sequence which is suitable for discriminatively identifying event TC1507 in plants, plant material, or in products such as, but not limited to, food or feed products (fresh or processed) comprising, or derived from plant material.

As used herein, the terms "insect resistant" and "impacting insect pests" refers to effecting changes in insect feeding, growth, and/or behavior at any stage of development, including but not limited to: killing the insect; retarding growth; preventing reproductive capability; and the like.

As used herein, the terms "pesticidal activity" and "insecticidal activity" are used synonymously to refer to activity of an organism or a substance (such as, for example, a protein) that can be measured by numerous parameters including, but not limited to, pest mortality, pest weight loss, pest attraction, pest repellency, and other behavioral and physical changes of a pest after feeding on and/or exposure to the organism or substance for an appropriate length of time. For example "pesticidal proteins" are proteins that display pesticidal activity by themselves or in combination with other proteins.

"Coding sequence" refers to a nucleotide sequence that codes for a specific amino acid sequence. As used herein, the terms "encoding" or "encoded" when used in the context of a specified nucleic acid mean that the nucleic acid comprises the requisite information to guide translation of the nucleotide sequence into a specified protein. The information by which a protein is encoded is specified by the use of codons. A nucleic acid encoding a protein may comprise non-translated sequences (e.g., introns) within translated regions of the nucleic acid or may lack such intervening non-translated sequences (e.g., as in cDNA).

"Gene" refers to a nucleic acid fragment that expresses a specific protein, including regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may

US 8,901,378 B2

7

comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. "Foreign" refers to material not normally found in the location of interest. Thus "foreign DNA" may comprise both recombinant DNA as well as newly introduced, rearranged DNA of the plant. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. The site in the plant genome where a recombinant DNA has been inserted may be referred to as the "insertion site" or "target site".

As used herein, "insert DNA" refers to the heterologous DNA within the expression cassettes used to transform the plant material while "flanking DNA" can exist of either genomic DNA naturally present in an organism such as a plant, or foreign (heterologous) DNA introduced via the transformation process which is extraneous to the original insert DNA molecule, e.g. fragments associated with the transformation event. A "flanking region" or "flanking sequence" as used herein refers to a sequence of at least 20 base pair, preferably at least 50 base pair, and up to 5000 base pair which is located either immediately upstream of and contiguous with or immediately downstream of and contiguous with the original foreign insert DNA molecule. Transformation procedures leading to random integration of the foreign DNA will result in transformants containing different flanking regions characteristic and unique for each transformant. When recombinant DNA is introduced into a plant through traditional crossing, its flanking regions will generally not be changed. Transformants will also contain unique junctions between a piece of heterologous insert DNA and genomic DNA, or 2 pieces of genomic DNA, or 2 pieces of heterologous DNA. A "junction" is a point where 2 specific DNA fragments join. For example, a junction exists where insert DNA joins flanking DNA. A junction point also exists in a transformed organism where 2 DNA fragments join together in a manner that is modified from that found in the native organism. "Junction DNA" refers to DNA that comprises a junction point.

As used herein, "heterologous" in reference to a nucleic acid is a nucleic acid that originates from a foreign species, or, if from the same species, is substantially modified from its native form in composition and/or genomic locus by deliberate human intervention. For example, a promoter operably linked to a heterologous nucleotide sequence can be from a species different from that from which the nucleotide sequence was derived, or, if from the same species, the promoter is not naturally found operably linked to the nucleotide sequence. A heterologous protein may originate from a foreign species, or, if from the same species, is substantially modified from its original form by deliberate human intervention.

"Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include promoters, translation leader sequences, introns, and polyadenylation recognition sequences.

8

"Promoter" refers to a nucleotide sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. The promoter sequence consists of proximal and more distal upstream elements, the latter elements are often referred to as enhancers. Accordingly, an "enhancer" is a nucleotide sequence that can stimulate promoter activity and may be an innate element of the promoter or a heterologous element inserted to enhance the level or tissue-specificity of a promoter. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic nucleotide segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters that cause a nucleic acid fragment to be expressed in most cell types at most times are commonly referred to as "constitutive promoters". New promoters of various types useful in plant cells are constantly being discovered; numerous examples may be found in the compilation by Okamuro and Goldberg (1989) *Biochemistry of Plants* 15:1-82. It is further recognized that since in most cases the exact boundaries of regulatory sequences have not been completely defined, nucleic acid fragments of different lengths may have identical promoter activity.

The "translation leader sequence" refers to a nucleotide sequence located between the promoter sequence of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect numerous parameters including, processing of the primary transcript to mRNA, mRNA stability and/or translation efficiency. Examples of translation leader sequences have been described (Turner and Foster (1995) *Mol. Biotechnol.* 3:225-236).

The "3' non-coding sequences" refer to nucleotide sequences located downstream of a coding sequence and include polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The use of different 3' non-coding sequences is exemplified by Ingelbrecht et al. (1989) *Plant Cell* 1:671-680.

A "protein" or "polypeptide" is a chain of amino acids arranged in a specific order determined by the coding sequence in a polynucleotide encoding the polypeptide.

A DNA construct is an assembly of DNA molecules linked together that provide one or more expression cassettes. The DNA construct may be a plasmid that is enabled for self replication in a bacterial cell and contains various endonuclease enzyme restriction sites that are useful for introducing DNA molecules that provide functional genetic elements, i.e., promoters, introns, leaders, coding sequences, 3' termination regions, among others; or a DNA construct may be a linear assembly of DNA molecules, such as an expression cassette. The expression cassette contained within a DNA construct comprise the necessary genetic elements to provide transcription of a messenger RNA. The expression cassette can be designed to express in prokaryote cells or eukaryotic cells. Expression cassettes of the present invention are designed to express most preferably in plant cells.

The DNA molecules of the invention are provided in expression cassettes for expression in an organism of interest. The cassette will include 5' and 3' regulatory sequences operably linked to a coding sequence of the invention. "Operably

US 8,901,378 B2

9

linked” means that the nucleic acid sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in the same reading frame. Operably linked is intended to indicate a functional linkage between a promoter and a second sequence, wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. The cassette may additionally contain at least one additional gene to be cotransformed into the organism. Alternatively, the additional gene(s) can be provided on multiple expression cassettes or multiple DNA constructs.

The expression cassette will include in the 5' to 3' direction of transcription: a transcriptional and translational initiation region, a coding region, and a transcriptional and translational termination region functional in the organism serving as a host. The transcriptional initiation region (i.e., the promoter) may be native or analogous, or foreign or heterologous to the host organism. Additionally, the promoter may be the natural sequence or alternatively a synthetic sequence. The expression cassettes may additionally contain 5' leader sequences in the expression cassette construct. Such leader sequences can act to enhance translation.

It is to be understood that as used herein the term “transgenic” includes any cell, cell line, callus, tissue, plant part, or plant the genotype of which has been altered by the presence of a heterologous nucleic acid including those transgenics initially so altered as well as those created by sexual crosses or asexual propagation from the initial transgenic. The term “transgenic” as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by conventional plant breeding methods or by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

A transgenic “event” is produced by transformation of plant cells with a heterologous DNA construct(s), including a nucleic acid expression cassette that comprises a transgene of interest, the regeneration of a population of plants resulting from the insertion of the transgene into the genome of the plant, and selection of a particular plant characterized by insertion into a particular genome location. An event is characterized phenotypically by the expression of the transgene. At the genetic level, an event is part of the genetic makeup of a plant. The term “event” also refers to progeny produced by a sexual outcross between the transformant and another variety that include the heterologous DNA. Even after repeated back-crossing to a recurrent parent, the inserted DNA and flanking DNA from the transformed parent is present in the progeny of the cross at the same chromosomal location. The term “event” also refers to DNA from the original transformant comprising the inserted DNA and flanking sequence immediately adjacent to the inserted DNA that would be expected to be transferred to a progeny that receives inserted DNA including the transgene of interest as the result of a sexual cross of one parental line that includes the inserted DNA (e.g., the original transformant and progeny resulting from selfing) and a parental line that does not contain the inserted DNA.

An insect resistant TC1507 corn plant can be bred by first sexually crossing a first parental corn plant consisting of a corn plant grown from the transgenic TC1507 corn plant and progeny thereof derived from transformation with the expression cassettes of the present invention that confers insect resistance, and a second parental corn plant that lacks insect resistance, thereby producing a plurality of first progeny plants; and then selecting a first progeny plant that is resistant to insects; and selfing the first progeny plant, thereby produc-

10

ing a plurality of second progeny plants; and then selecting from the second progeny plants an insect resistant plant. These steps can further include the back-crossing of the first insect resistant progeny plant or the second insect resistant progeny plant to the second parental corn plant or a third parental corn plant, thereby producing a corn plant that is resistant to insects.

As used herein, the term “plant” includes reference to whole plants, plant organs (e.g., leaves, stems, roots, etc.), seeds, plant cells, and progeny of same. Parts of transgenic plants understood to be within the scope of the invention comprise, for example, plant cells, protoplasts, tissues, callus, embryos as well as flowers, stems, fruits, leaves, and roots originating in transgenic plants or their progeny previously transformed with a DNA molecule of the invention and therefore consisting at least in part of transgenic cells, are also an aspect of the present invention.

As used herein, the term “plant cell” includes, without limitation, seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, roots, shoots, gametophytes, sporophytes, pollen, and microspores. The class of plants that can be used in the methods of the invention is generally as broad as the class of higher plants amenable to transformation techniques, including both monocotyledonous and dicotyledonous plants.

“Transformation” refers to the transfer of a nucleic acid fragment into the genome of a host organism, resulting in genetically stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as “transgenic” organisms. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation (De Blaere et al. (1987) *Meth. Enzymol.* 143:277) and particle-accelerated or “gene gun” transformation technology (Klein et al. (1987) *Nature* (London) 327:70-73; U.S. Pat. No. 4,945,050, incorporated herein by reference). Additional transformation methods are disclosed below.

Thus, isolated polynucleotides of the present invention can be incorporated into recombinant constructs, typically DNA constructs, which are capable of introduction into and replication in a host cell. Such a construct can be a vector that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. A number of vectors suitable for stable transfection of plant cells or for the establishment of transgenic plants have been described in, e.g., Pouwels et al., (1985; Supp. 1987) *Cloning Vectors: A Laboratory Manual*, Weissbach and Weissbach (1989) *Methods for Plant Molecular Biology*, (Academic Press, New York); and Flevin et al., (1990) *Plant Molecular Biology Manual*, (Kluwer Academic Publishers). Typically, plant expression vectors include, for example, one or more cloned plant genes under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker. Such plant expression vectors also can contain a promoter regulatory region (e.g., a regulatory region controlling inducible or constitutive, environmentally- or developmentally-regulated, or cell- or tissue-specific expression), a transcription initiation start site, a ribosome binding site, an RNA processing signal, a transcription termination site, and/or a polyadenylation signal.

It is also to be understood that two different transgenic plants can also be mated to produce offspring that contain two independently segregating added, exogenous genes. Selfing of appropriate progeny can produce plants that are homozygous for both added, exogenous genes. Back-crossing to a parental plant and out-crossing with a non-transgenic plant are also contemplated, as is vegetative propagation. Descriptions of other breeding methods that are commonly used for

US 8,901,378 B2

11

different traits and crops can be found in one of several references, e.g., Fehr, in *Breeding Methods for Cultivar Development*, Wilcos J. ed., American Society of Agronomy, Madison Wis. (1987).

A “probe” is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. Such a probe is complementary to a strand of a target nucleic acid, in the case of the present invention, to a strand of isolated DNA from corn event TC1507 whether from a corn plant or from a sample that includes DNA from the event. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

“Primers” are isolated nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand, then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs of the present invention refer to their use for amplification of a target nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods. “PCR” or “polymerase chain reaction” is a technique used for the amplification of specific DNA segments (see, U.S. Pat. Nos. 4,683,195 and 4,800,159; herein incorporated by reference).

Probes and primers are of sufficient nucleotide length to bind to the target DNA sequence specifically in the hybridization conditions or reaction conditions determined by the operator. This length may be of any length that is of sufficient length to be useful in a detection method of choice. Generally, 11 nucleotides or more in length, preferably 18 nucleotides or more, and more preferably 22 nucleotides or more, are used. Such probes and primers hybridize specifically to a target sequence under high stringency hybridization conditions. Preferably, probes and primers according to the present invention have complete DNA sequence similarity of contiguous nucleotides with the target sequence, although probes differing from the target DNA sequence and that retain the ability to hybridize to target DNA sequences may be designed by conventional methods. Probes can be used as primers, but are generally designed to bind to the target DNA or RNA and not be used in an amplification process.

Specific primers can be used to amplify an integration fragment to produce an amplicon that can be used as a “specific probe” for identifying event TC1507 in biological samples. When the probe is hybridized with the nucleic acids of a biological sample under conditions which allow for the binding of the probe to the sample, this binding can be detected and thus allow for an indication of the presence of event TC1507 in the biological sample. Such identification of a bound probe has been described in the art. The specific probe is preferably a sequence which, under optimized conditions, hybridizes specifically to a region within the 5' or 3' flanking region of the event and preferably also comprises a part of the foreign DNA contiguous therewith. Preferably the specific probe comprises a sequence of at least 80%, preferably between 80 and 85%, more preferably between 85 and 90%, especially preferably between 90 and 95%, and most preferably between 95 and 100% identical (or complementary) to a specific region of the event.

Methods for preparing and using probes and primers are described, for example, in *Molecular Cloning: A Laboratory Manual*, 2<sup>nd</sup> ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. 1989

12

(hereinafter, “Sambrook et al., 1989”); *Current Protocols in Molecular Biology*, ed. Ausubel et al., Greene Publishing and Wiley-Interscience, New York, 1992 (with periodic updates) (hereinafter, “Ausubel et al., 1992”); and Innis et al., *PCR Protocols: A Guide to Methods and Applications*, Academic Press: San Diego, 1990. PCR primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as the PCR primer analysis tool in Vector NTI version 6 (Informax Inc., Bethesda Md.); PrimerSelect (DNASTAR Inc., Madison, Wis.); and Primer (Version 0.5, © 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.). Additionally, the sequence can be visually scanned and primers manually identified using guidelines known to one of skill in the art.

A “kit” as used herein refers to a set of reagents for the purpose of performing the method of the invention, more particularly, the identification of the event TC1507 in biological samples. The kit of the invention can be used, and its components can be specifically adjusted, for purposes of quality control (e.g. purity of seed lots), detection of event TC1507 in plant material, or material comprising or derived from plant material, such as but not limited to food or feed products. “Plant material” as used herein refers to material which is obtained or derived from a plant.

Primers and probes based on the flanking DNA and insert sequences disclosed herein can be used to confirm (and, if necessary, to correct) the disclosed sequences by conventional methods, e.g., by re-cloning and sequencing such sequences. The nucleic acid probes and primers of the present invention hybridize under stringent conditions to a target DNA sequence. Any conventional nucleic acid hybridization or amplification method can be used to identify the presence of DNA from a transgenic event in a sample. Nucleic acid molecules or fragments thereof are capable of specifically hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of specifically hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure.

A nucleic acid molecule is said to be the “complement” of another nucleic acid molecule if they exhibit complete complementarity. As used herein, molecules are said to exhibit “complete complementarity” when every nucleotide of one of the molecules is complementary to a nucleotide of the other. Two molecules are said to be “minimally complementary” if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional “low-stringency” conditions. Similarly, the molecules are said to be “complementary” if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional “high-stringency” conditions. Conventional stringency conditions are described by Sambrook et al., 1989, and by Haymes et al., In: *Nucleic Acid Hybridization, a Practical Approach*, IRL Press, Washington, D.C. (1985), departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. In order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

In hybridization reactions, specificity is typically the function of post-hybridization washes, the critical factors being the ionic strength and temperature of the final wash solution. The thermal melting point (T<sub>m</sub>) is the temperature (under defined ionic strength and pH) at which 50% of a comple-



US 8,901,378 B2

13

mentary target sequence hybridizes to a perfectly matched probe. For DNA-DNA hybrids, the  $T_m$  can be approximated from the equation of Meinkoth and Wahl (1984) *Anal. Biochem.* 138:267-284:  $T_m = 81.5^\circ \text{C} + 16.6 (\log M) + 0.41 (\% \text{GC}) - 0.61 (\% \text{ form}) - 500/L$ ; where  $M$  is the molarity of monovalent cations,  $\% \text{GC}$  is the percentage of guanosine and cytosine nucleotides in the DNA,  $\% \text{ form}$  is the percentage of formamide in the hybridization solution, and  $L$  is the length of the hybrid in base pairs.  $T_m$  is reduced by about  $1^\circ \text{C}$ . for each 1% of mismatching; thus,  $T_m$ , hybridization, and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with  $>90\%$  identity are sought, the  $T_m$  can be decreased  $10^\circ \text{C}$ . Generally, stringent conditions are selected to be about  $5^\circ \text{C}$ . lower than the  $T_m$  for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or  $4^\circ \text{C}$ . lower than the  $T_m$ ; moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or  $10^\circ \text{C}$ . lower than the  $T_m$ ; low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or  $20^\circ \text{C}$ . lower than the  $T_m$ .

Using the equation, hybridization and wash compositions, and desired  $T_m$ , those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a  $T_m$  of less than  $45^\circ \text{C}$ . (aqueous solution) or  $32^\circ \text{C}$ . (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen (1993) *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes*, Part I, Chapter 2 (Elsevier, New York); and Ausubel et al., eds. (1995) *Current Protocols in Molecular Biology*, Chapter 2 (Greene Publishing and Wiley-Interscience, New York). See Sambrook et al. (1989) *Molecular Cloning: A Laboratory Manual* (2d ed., Cold Spring Harbor Laboratory Press, Plainview, N.Y.).

As used herein, a substantially homologous sequence is a nucleic acid molecule that will specifically hybridize to the complement of the nucleic acid molecule to which it is being compared under high stringency conditions. Appropriate stringency conditions which promote DNA hybridization, for example,  $6\times$  sodium chloride/sodium citrate (SSC) at about  $45^\circ \text{C}$ ., followed by a wash of  $2\times \text{SSC}$  at  $50^\circ \text{C}$ ., are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about  $30^\circ \text{C}$ . for short probes (e.g., 10 to 50 nucleotides) and at least about  $60^\circ \text{C}$ . for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of a destabilizing agent such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at  $37^\circ \text{C}$ ., and a wash in  $1\times$  to  $2\times \text{SSC}$  ( $20\times \text{SSC} = 3.0 \text{ M NaCl}/0.3 \text{ M trisodium citrate}$ ) at 50 to  $55^\circ \text{C}$ . Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at  $37^\circ \text{C}$ ., and a wash in  $0.5\times$  to  $1\times \text{SSC}$  at 55 to  $60^\circ \text{C}$ . Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at  $37^\circ \text{C}$ ., and a wash in  $0.1\times \text{SSC}$  at 60 to  $65^\circ \text{C}$ . In a preferred embodiment, a nucleic acid of the present invention will specifically hybridize to one or more of

14

the nucleic acid molecules unique to the TC1507 event or complements thereof or fragments of either under moderately stringent conditions.

Methods of alignment of sequences for comparison are well known in the art. Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. Non-limiting examples of such mathematical algorithms are the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17; the local homology algorithm of Smith et al. (1981) *Adv. Appl. Math.* 2:482; the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48:443-453; the search-for-similarity-method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci.* 85:2444-2448; the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877.

Computer implementations of these mathematical algorithms can be utilized for comparison of sequences to determine sequence identity. Such implementations include, but are not limited to: CLUSTAL in the PC/Gene program (available from Intelligenetics, Mountain View, Calif.); the ALIGN program (Version 2.0); the ALIGN PLUS program (version 3.0, copyright 1997); and GAP, BESTFIT, BLAST, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Version 10 (available from Accelrys, 9685 Scranton Road, San Diego, Calif. 92121, USA). Alignments using these programs can be performed using the default parameters.

The CLUSTAL program is well described by Higgins and Sharp, *Gene* 73: 237-244 (1988); Higgins and Sharp, *CABIOS* 5: 151-153 (1989); Corpet, et al., *Nucleic Acids Research* 16: 10881-90 (1988); Huang, et al., *Computer Applications in the Biosciences* 8: 155-65 (1992), and Pearson, et al., *Methods in Molecular Biology* 24: 307-331 (1994). The ALIGN and the ALIGN PLUS programs are based on the algorithm of Myers and Miller (1988) supra. The BLAST programs of Altschul et al. (1990) *J. Mol. Biol.* 215:403 are based on the algorithm of Karlin and Altschul (1990) supra. The BLAST family of programs which can be used for database similarity searches includes: BLASTN for nucleotide query sequences against nucleotide database sequences; BLASTX for nucleotide query sequences against protein database sequences; BLASTP for protein query sequences against protein database sequences; TBLASTN for protein query sequences against nucleotide database sequences; and TBLASTX for nucleotide query sequences against nucleotide database sequences. See, *Current Protocols in Molecular Biology*, Chapter 19, Ausubel, et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). Alignment may also be performed manually by inspection.

To obtain gapped alignments for comparison purposes, Gapped BLAST (in BLAST 2.0) can be utilized as described in Altschul et al. (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-BLAST (in BLAST 2.0) can be used to perform an iterated search that detects distant relationships between molecules. See Altschul et al. (1997) supra. When utilizing BLAST, Gapped BLAST, PSI-BLAST, the default parameters of the respective programs (e.g., BLASTN for nucleotide sequences, BLASTX for proteins) can be used. See [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov).

As used herein, "sequence identity" or "identity" in the context of two nucleic acid or polypeptide sequences makes reference to the residues in the two sequences that are the same when aligned for maximum correspondence over a specified comparison window. When percentage of sequence identity is used in reference to proteins it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues

US 8,901,378 B2

15

are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. When sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences that differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity. Thus, for example, where an identical amino acid is given a score of 1 and a non-conservative substitution is given a score of zero, a conservative substitution is given a score between zero and 1. The scoring of conservative substitutions is calculated, e.g., as implemented in the program PC/GENE (Intelligenetics, Mountain View, Calif.).

As used herein, "percentage of sequence identity" means the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison, and multiplying the result by 100 to yield the percentage of sequence identity.

Regarding the amplification of a target nucleic acid sequence (e.g., by PCR) using a particular amplification primer pair, "stringent conditions" are conditions that permit the primer pair to hybridize only to the target nucleic-acid sequence to which a primer having the corresponding wild-type sequence (or its complement) would bind and preferably to produce a unique amplification product, the amplicon, in a DNA thermal amplification reaction.

The term "specific for (a target sequence)" indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

As used herein, "amplified DNA" or "amplicon" refers to the product of nucleic acid amplification of a target nucleic acid sequence that is part of a nucleic acid template. For example, to determine whether a corn plant resulting from a sexual cross contains transgenic event genomic DNA from the corn plant of the present invention, DNA extracted from the corn plant tissue sample may be subjected to a nucleic acid amplification method using a DNA primer pair that includes a first primer derived from flanking sequence adjacent to the insertion site of inserted heterologous DNA, and a second primer derived from the inserted heterologous DNA to produce an amplicon that is diagnostic for the presence of the event DNA. Alternatively, the second primer may be derived from the flanking sequence. The amplicon is of a length and has a sequence that is also diagnostic for the event. The amplicon may range in length from the combined length of the primer pairs plus one nucleotide base pair to any length of amplicon producible by a DNA amplification protocol. Alternatively, primer pairs can be derived from flanking sequence on both sides of the inserted DNA so as to produce an amplicon that includes the entire insert nucleotide sequence of the PHI8999A expression construct, see FIG. 1, approximately 6.2 Kb in size. A member of a primer pair derived from the

16

flanking sequence may be located a distance from the inserted DNA sequence, this distance can range from one nucleotide base pair up to the limits of the amplification reaction, or about twenty thousand nucleotide base pairs. The use of the term "amplicon" specifically excludes primer dimers that may be formed in the DNA thermal amplification reaction.

Nucleic acid amplification can be accomplished by any of the various nucleic acid amplification methods known in the art, including the polymerase chain reaction (PCR). A variety of amplification methods are known in the art and are described, inter alia, in U.S. Pat. Nos. 4,683,195 and 4,683,202 and in *PCR Protocols: A Guide to Methods and Applications*, ed. Innis et al., Academic press, San Diego, 1990. PCR amplification methods have been developed to amplify up to 22 Kb of genomic DNA and up to 42 Kb of bacteriophage DNA (Cheng et al., *Proc. Natl. Acad. Sci. USA* 91:5695-5699, 1994). These methods as well as other methods known in the art of DNA amplification may be used in the practice of the present invention. It is understood that a number of parameters in a specific PCR protocol may need to be adjusted to specific laboratory conditions and may be slightly modified and yet allow for the collection of similar results. These adjustments will be apparent to a person skilled in the art.

The amplicon produced by these methods may be detected by a plurality of techniques, including, but not limited to, Genetic Bit Analysis (Nikiforov, et al. *Nucleic Acid Res.* 22:4167-4175, 1994) where a DNA oligonucleotide is designed which overlaps both the adjacent flanking DNA sequence and the inserted DNA sequence. The oligonucleotide is immobilized in wells of a microwell plate. Following PCR of the region of interest (using one primer in the inserted sequence and one in the adjacent flanking sequence) a single-stranded PCR product can be hybridized to the immobilized oligonucleotide and serve as a template for a single base extension reaction using a DNA polymerase and labeled ddNTPs specific for the expected next base. Readout may be fluorescent or ELISA-based. A signal indicates presence of the insert/flanking sequence due to successful amplification, hybridization, and single base extension.

Another detection method is the Pyrosequencing technique as described by Winge (*Innov. Pharma. Tech.* 00: 18-24, 2000). In this method an oligonucleotide is designed that overlaps the adjacent DNA and insert DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (one primer in the inserted sequence and one in the flanking sequence) and incubated in the presence of a DNA polymerase, ATP, sulfurylase, luciferase, apyrase, adenosine 5' phosphosulfate and luciferin. dNTPs are added individually and the incorporation results in a light signal which is measured. A light signal indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single or multi-base extension.

Fluorescence Polarization as described by Chen et al., (*Genome Res.* 9:492-498, 1999) is a method that can be used to detect an amplicon of the present invention. Using this method an oligonucleotide is designed which overlaps the flanking and inserted DNA junction. The oligonucleotide is hybridized to a single-stranded PCR product from the region of interest (one primer in the inserted DNA and one in the flanking DNA sequence) and incubated in the presence of a DNA polymerase and a fluorescent-labeled ddNTP. Single base extension results in incorporation of the ddNTP. Incorporation can be measured as a change in polarization using a fluorometer. A change in polarization indicates the presence of the transgene insert/flanking sequence due to successful amplification, hybridization, and single base extension.



US 8,901,378 B2

17

Taqman® (PE Applied Biosystems, Foster City, Calif.) is described as a method of detecting and quantifying the presence of a DNA sequence and is fully understood in the instructions provided by the manufacturer. Briefly, a FRET oligonucleotide probe is designed which overlaps the flanking and insert DNA junction. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking genomic sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Hybridization of the FRET probe results in cleavage and release of the fluorescent moiety away from the quenching moiety on the FRET probe. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

Molecular Beacons have been described for use in sequence detection as described in Tyangi et al. (*Nature Biotech.* 14:303-308, 1996). Briefly, a FRET oligonucleotide probe is designed that overlaps the flanking and insert DNA junction. The unique structure of the FRET probe results in it containing secondary structure that keeps the fluorescent and quenching moieties in close proximity. The FRET probe and PCR primers (one primer in the insert DNA sequence and one in the flanking sequence) are cycled in the presence of a thermostable polymerase and dNTPs. Following successful PCR amplification, hybridization of the FRET probe to the target sequence results in the removal of the probe secondary structure and spatial separation of the fluorescent and quenching moieties. A fluorescent signal results. A fluorescent signal indicates the presence of the flanking/transgene insert sequence due to successful amplification and hybridization.

A hybridization reaction using a probe specific to a sequence found within the amplicon is yet another method used to detect the amplicon produced by a PCR reaction.

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention, in addition to those shown and described herein, will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

The disclosure of each reference set forth herein is incorporated herein by reference in its entirety.

## EXAMPLES

## Example 1

Transformation of Maize by Particle Bombardment and Regeneration of Transgenic Plants Containing the Cry1F Gene

A DNA molecule of 6.2 Kb, designated PHI8999A (see FIG. 1 and SEQ ID NO:25), which includes a first transgene expression cassette comprising the promoter, 5' untranslated exon, and first intron of the maize ubiquitin (Ubi-1) gene (Christensen et al. (1992) *Plant Mol. Biol.* 18:675-689 and Christensen and Quail (1996) *Transgenic Res.* 5:213-218) operably connected to a DNA molecule encoding a *Bacillus thuringiensis*  $\delta$ -endotoxin identified as Cry1F (U.S. Pat. Nos. 5,188,960 and 6,218,188) operably connected to a DNA molecule comprising a 3' ORF25 transcriptional terminator iso-

18

lated from *Agrobacterium tumefaciens* (Barker et al. (1983) *Plant Mol. Biol.* 2:335-350), and a second transgene expression cassette comprising a DNA molecule of the cauliflower mosaic virus (CaMV) 35S promoter (Odell J. T. et al. (1985) *Nature* 313: 810-812; Mitsuhashi et al. (1996) *Plant Cell Physiol.* 37:49-59) operably connected to a DNA molecule encoding the selectable marker, phosphinothricin acetyltransferase (PAT) gene (Wohllleben W. et al. (1988) *Gene* 70:25-37) operably connected to a DNA molecule comprising a 3' transcriptional terminator from (CaMV) 35S (see Mitsuhashi et al. (1996) *Plant Cell Physiol.* 37:49-59) was used to transform maize embryo tissue.

B.t. Cry1F maize plants were obtained by microprojectile bombardment using the Biolistics® PDS-1000He particle gun manufactured by Bio-Rad, Hercules, Calif.; essentially as described by Klein et al. (1987) *Nature*, UK 327(6117): 70-73. Immature embryos isolated from maize ears, harvested soon after pollination were cultured on callus initiation medium for several days. On the day of transformation, microscopic tungsten particles were coated with purified PHI8999A DNA (SEQ ID NO:25) and accelerated into the cultured embryos, where the insert DNA was incorporated into the cell chromosome. Only insert PHI8999A was used during transformation and no additional plasmid DNA was incorporated into the transformant. After bombardment, embryos were transferred to callus initiation medium containing glufosinate as the selection agent. Individual embryos were kept physically separate during culture, and the majority of explants died on the selective medium.

Those embryos that survived and produced healthy, glufosinate-resistant callus tissue were assigned unique identification codes representing putative transformation events, and continually transferred to fresh selection medium. Plants were regenerated from tissue derived from each unique event and transferred to the greenhouse. Leaf samples were taken for molecular analysis to verify the presence of the transgene by PCR and to confirm expression of the Cry1F protein by ELISA. Plants were then subjected to a whole plant bioassay using European corn borer insects. Positive plants were crossed with inbred lines to obtain seed from the initial transformed plants. A number of lines were evaluated in the field. The TC1507 event was selected from a population of independent transgenic events based on a superior combination of characteristics, including insect resistance and agronomic performance (see Bing J W et al. (2000) Efficacy of Cry1F Transgenic Maize, 14<sup>th</sup> Biennial International Plant Resistance to Insects Workshop, Fort Collins, Colo., herein incorporated by reference).

## Example 2

Identification of Nucleotides Comprising the Flanking Sequence 5' to the Transgenic Insert DNA in *Bacillus thuringiensis* Cry1F Maize Line TC1507

To identify a DNA fragment that included sequence 5' to the PHI8999A insert in event TC1507, Spe I restriction enzyme fragments from event TC1507 genomic DNA were size selected on agarose gels, purified, and screened by Southern analysis to confirm hybridization to a Cry1F probe. Following confirmation of hybridization and fragment size, the fragments of interest were cloned into a pBluescript II SK (+)<sup>TM</sup> cloning vector to prepare an enriched size selected plasmid based genomic DNA library. A probe homologous to a portion of the Cry1F gene was used to screen the plasmid library for positive clones. A positive clone was identified, purified by additional screening, and confirmed to result in a

## US 8,901,378 B2

## 19

positive signal when hybridized to the Cry1F probe. Nearly 3 Kb of the Spe I fragment contained in the isolated positive clone was sequenced using a primer walking approach. To initiate the first sequencing run, a primer that binds to a known sequence in the cloning vector DNA was designed to sequence a portion of the DNA of interest. A second sequencing run over the same region using another primer oriented in the reverse direction provided second strand coverage. Primer walking was accomplished by repeatedly using sequence data from previous runs to design new primers that were then used to extend the next round of sequencing further into the DNA of interest until the flanking sequence 5' to the inserted transgenic DNA in maize event TC1507 was obtained. Specific sequence information is provided in Example 4.

## Example 3

Confirmation of Flanking Sequence 5' to the B.t. Cry1F Maize Line TC1507 Insert

To confirm the 5' flanking sequence of the B.t. Cry1F maize line TC1507 insert, PCR primer pairs were designed to obtain overlapping PCR products extending from the 5' flanking region into the full-length PHI8999A transgenic insert. PCR

## 20

products were successfully amplified from B.t. Cry1F maize line TC1507 genomic DNA, isolated, and sequenced for Region 1 through Region 6, shown in Table 1, and confirmed to match the previously determined sequence from the Spe I fragment, described in Example 2. However, the region from by 2358 to by 2829, immediately adjacent and 5' to the start of the full-length insert was recalcitrant to PCR amplification and appeared to be larger than the sequence obtained from the Spe I clone described above. The use of primer pairs flanking this region and the Advantage®-GC 2 Polymerase Mix (BD Biosciences Clontech, Palo Alto, Calif.) was successful in amplifying PCR products from B.t. Cry1F maize line TC1507 genomic DNA for sequencing. The amplification conditions used to produce amplicons with the Advantage®-GC 2 system are shown in Table 10. The DNA primer pairs used to confirm the sequence in the region from by 2358 to 2829 are those listed in SEQ ID NO:1 and SEQ ID NO:2; and SEQ ID NO:2 and SEQ ID NO:23. Sequence from this region is described in Table 1 (Regions 7a, 7b, 7c, and 8).

## Example 4

Event TC1507 5' Flanking Sequence

A description of each region is provided in Table 1.

Region 1 Maize genomic (no significant homology) (SEQ ID NO: 28)

1 ACTAGTTTCC TAGCCCGCGT CGTGCCCTA CCCACCGAC GTTTATGGAA

51 GGTGCCATT CACGGTTCTT CGTGGCCGCC CCTAAGGATG TAAATGGTCG

101 GTAAATCCG GTAAATTCC GGTACCGTTT ACCAGATTTT TCCAGCCGTT

151 TTCGGATTTA TCGGGATATA CAGAAAACGA GACGGAACG GAATAGGTTT

201 TTTTTCGAAA ACGGTACGGT AACGGTGAG ACAAATTAC CGTCCGTTTT

251 CGTATTTCTC GGGAACTCT GGTATATTCC CGTATTTGTC CCGTATTTTC

301 CCGACCCACG GACCTGCCAA TCAACCATCA GCCAGTCAGC CCATCCCCAC

351 AGCTATGGCC CATGGGGCCA TGTGGCCAC ATGCCACGC AACGCAAGGC

401 AGTAAGGCTG GCAGCCTGGC ACGCATTGAC GCATGTGGAC ACACACAGCC

451 GCCGCCTGTT CGTGTTTCTG TGCCGTTGTG CGAGACTGTG ACTGCGAGTG

501 GCGGAGTCGG CGAACGGCGA GGCCTCTCCG GAGTCTGGAC TGCGGCTGTG

551 GACAGCGACG CTGTGACGGC GACTCGGCGA AGCCCCAAGC TACCAAGCCC

601 CCAAGTCCCC ATCCATCTCT GCTTCTCTGG TCATCTCCTT CCCCTGGTGC

651 ATCTGCAGGC GCCAGACCG

Region 2 Undescribed maize genomic sequence (complement) (SEQ ID NO: 29)

670 G CCGAAGCATC ACGAAACGCA CTAAGACCTC

701 GAAGGAGTCA AACCCTCCT CCGAGGCTC GGGGGCTACA CCCGCGGGT

751 GCGCTCGCGC GCACCCACCG GAACAAAATG TAACCGAGAA AGGTGCGTCC

801 CCTTGCAAAA AAAGTGCAGC AAAAGCCTCC AAGCGAGTAT TAACACTCAC

851 TTGAGGCTC GGGGGCTAC

Region 3 Fragment of maize Huck-1 retrotransposon (SEQ ID NO: 30)

870 T GTCGGGGACC ATAATTAGGG GTACCCCAA

901 GACTCCTAAT CTCAGCTGGT AACCCCATC AGCACAAAGC TGCAAAGGCC

951 TGATGGGTGC GATTAAGTCA AGGCTCGGTC CACTCAAGGG ACACGATCTC

US 8,901,378 B2

21

22

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1001 GCCTCGCCCC AGCCCAGCCT CGGGCAAGGG CGGCCGACCC CGAGGATTCA  
1051 CGTCTCGCCC GAGGGCCCCC TCAAGCGACG GGCACACCTT CGGCTCGCCC  
1101 GAGGCCCATC CTTCGCCGAG AAGCAACCTT GGCCAGATCG CCACACCGAC  
1151 CGACCGTATC GCAGGAGCAT TTAATGCGAG GATCGCCTGA CACCTTATCC  
1201 TGACGCGCGC TCTTCAGTCG ACAGAGCCGA AGTGACCGCA ATCACTTCGC  
1251 CGCTCCACTG ACCGACCTGA CAAGAAGACA GCGCCGCTG CGTCGCTCCG  
1301 ACTGCTGTGC CACTCGACAG AGTGAGGCTG ACAGCAGCCA AGTCCGGCCT  
1351 CGGGCGCCAT AGGAAGCTCC GCCTCGCCCG ACCCTAGGGC TCGGACTCGG  
1401 CCTCGGCTCC GGAAGACGAC GAACTACGCT TCGCCCGACC CCAGGGCTTG  
1451 GACTCAGCCT CGGCTCCGGA AGACGACGAA TTCCGCCTCG CCCGACCCCA  
1501 GGGCTCGGAC TCGGCCTCGG CTCCAGAAGA CGACGAACTC CGCCTCGCCC  
1551 GACCCAGGG CTGGACTCA GCCTCGGCTC CGGAAGACGA CGAACTCCGC  
1601 CTCGCCCAGC CCCAGGGCTC GGACTCAGCC TCGGCCTCAG ACGATGGTCT  
1651 CCGCCTCGCC CGACCCGGGG CTCGGACTCG A

Region 4 Fragment of cry1F gene

(SEQ ID NO: 31)

1682 CCTTTCTAT CGGACCTTGT  
1701 CAGATCCTGT CTTGTCCTGA GGAGGCTTTG GCAATCCTCA CTATGTACTC  
1751 GGTCTTAGGG GAGTGGCCTT TCAACAACT GGTACGAATC ACACCCGCAC  
1801 ATTCAGGAAC TCCGGGACCA TTGACTCTCT AGATGAGATA CCACCTCAAG  
1851 ACAACAGCGG CGCACCTTGG AATGACTACT CCCATGTGCT GAATCATGTT  
1901 ACCTTTGTGC GCTGGCCAGG TGAGATCTCA GGTTCGACT CATGGAGAGC  
1951 ACCAATGTTT TCTTGACGC ATCGTAGCGC TACCCCCACA AACACCATTG  
2001 ATCCAGAGAG AATCAC

Region 5 Fragment of maize chloroplast rpoC2 gene

(SEQ ID NO: 32)

2017 TCAT TCTTCAAGAA CTGCATATCT TGCCGAGATC  
2051 CTCATCCCTA AAGGTACTTG ACAATAGTAT TATTGGAGTC GATACACAAC  
2101 TCACAAAAA TACAAGAAGT CGACTAGGTG GATTGGTCCG AGTGAAGAGA  
2151 AAAAAAAGCC ATACAGAACT CAAAATCTTT TCCGAGATA TTCATTTTCC  
2201 TGAAGAGGCG GATAAGATAT TAGGTGGCAG TTTGATACCA CCAGAAAGAG  
2251 AAAAAAAGA TTCTAAGGAA TCAAAAAA GGAAAAATTG GGTTTATGTT  
2301 CAACGAAAA AATTTCTCAA AAGCAAGGAA AAGTATT

Region 6 Fragment of maize chloroplast or ubiZM1(2) promoter

(SEQ ID NO: 33)

2338 GTG GCTATTTATC

2351 TATC

Nucleotides 2355-2358 (CGT) connect Region 6 to Region 7a.

Region 7a Fragment of pat gene

(SEQ ID NO: 34)

2358 GCA GCTGATATGG CCGCGGTTTG TGATATCGTT AACCATTACA  
2401 TTGAGACGTC TACAGTGAAC TTTAGGACAG AGCCACAAAC ACCACAAGAG  
2451 TGGATTGATG ATCTAGAGAG GTTGCAAGAT AGATACCCTT GGTTGGTTGC  
2501 TGAGGTTGAG GGTGTTGTGG CTGGTATTGC TTACGCTGGG CCCTGGAAGG  
2551 CTAGGAAC

## US 8,901,378 B2

23

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Region 7b Fragment of pat gene (complement)  
(SEQ ID NO: 35)

2559 CC TCAACCTCAG CAACCAACCA ATGGTATCTA TCTTGCAACC

2601 TCTCTAGATC ATCAATCCAC TCTTGTGGTG TTTGTGGCTC TGTCCTAAAG

2651 TTCACTGTAG ACGTCTCAAT GTAATGGTTA ACGATATCAC AAACCG

Region 7c Fragment of cry1F gene (complement)  
(SEQ ID NO: 36)

2697 AGAG

2701 AAGAGGGATC T

Region 8 Fragment of Polylinker  
(SEQ ID NO: 37)

2712 CGAAGCTTC GGCCGGGGCC CATCGATATC CGCGGGCATG

2751 CCTGCAGTGC AGCGTGACCC GGTCGTGCCC CTCTCTAGAG ATAATGAGCA

2801 TTGCATGTCT AAGTTATAAA AAATTACCA

Region 9 Full-length insert of PHI8999A  
(SEQ ID NO: 25)

24

## Example 5

Description of the Flanking Sequence 5' to the Insert  
in Maize Event TC1507

In order to more fully describe the event TC1507 5' flanking sequence, homology searching was done against the GenBank public databases (release 122, 2/01) using the Basic Local Alignment Search Tool (BLAST). The BLAST program performs sequence similarity searching and is particularly useful for identifying homologs to an unknown sequence. In addition to searching the public databases, pairwise alignments were performed using AlignX (InforMax Inc., Bethesda, Md.) to look for homology between the maize event TC1507 flanking sequence and the PHI8999A transgenic insert. The results of these homology searches are presented in Table 1. The TC1507 5' flanking sequence is numbered with base 1 being the furthest 5' to the insert and base 2830 at the starting point of the full-length PHI8999A trans-

genic insert (see FIG. 1). The percent identity values indicate the percentage of identical matches across the length of the sequences analyzed.

In most cases, similarity searching with the event TC1507 5' flanking sequence resulted in a match to one unique sequence based on a very high percent identity value. Those sequences are identified in Table 1. In addition, there are two regions in the TC1507 5' DNA flanking sequence with high similarity to more than one known sequence. In regions 870-1681 and 2338-2354, the percent identity scores with both sequence fragments are sufficiently high that a single match (homolog) cannot be determined. The two possible homologs for each of these regions are indicated in Table 1.

Highly similar sequences were identified for all but the first 669 base pairs of sequence. Generally, the results of similarity searching indicate high homology with maize genomic sequences 5' to base 1681. The region from base 1682 to the start of the PHI8999A insert at position 2830 contains some fragments associated with the transformation event.

TABLE 1

Sequence summary for event TC1507 insert						
Region	Location in SEQ ID NO: 24	Size bp	% Identity	Homolog	Location in homologous sequence	Description
1	1-669	669	N/A <sup>1</sup>	N/A	N/A	No significant homology detected
2	670-869	200	90.5	AF123535	52432-52632 (complement)	Undescribed maize genomic sequence
3	870-1681	812	89.4	AF050439	1-801	Fragment of maize Huck-1 retrotransposon 5' LTR <sup>2</sup>
			86.6	AF050438	1-797	Fragment of maize Huck-1 retrotransposon 3' LTR
4	1682-2016	335	100.0	PHI8999A	3149-3483	Fragment of cry1F gene
5	2017-2337	321	100.0	X86563	29429-29749	Fragment of maize chloroplast rpoC2 gene (RNA polymerase beta-2 subunit)

US 8,901,378 B2

25

26

TABLE 1-continued

Sequence summary for event TC1507 insert						
Region	Location in SEQ ID NO: 24	Size bp	% Identity	Homolog	Location in homologous sequence	Description
6	2338-2354	17	100.0	X86563	97643-97659	Fragment of maize chloroplast trnI gene (tRNA-Ile)
			82.4	PHI8999A	182-197	Fragment of maize ubiZM1(2) promoter
7a	2358-2558	201	100.0	PHI8999A	5320-5475	Fragment of pat gene
7b	2559-2696	138	99	PHI8999A	5336-5518 (complement)	Fragment of pat gene
7c	2697-2711	15	100.0	PHI8999A	2544-2558 (complement)	Fragment of cry1F gene
8	2712-2829	118	100.0	PHI8999A	36-153	Fragment of polylinker region (bases 36-80) and ubiZM1(2) promoter (bases 81-153)
9	2830-9015	6186	100.0	PHI8999A	11-6196	Full-length insert of PHI8999A
10	9016-9565	550	100.0	PHI8999A	3906-4456 (complement)	Inverted ORF25 terminator
11	9566-9693	128	100.0	NC_001666	121851-121978 (complement) & 100759-100886	Fragment of maize chloroplast rps12 rRNA (23S ribosomal RNA)
12	9696-10087	392	99	NC_001666	17091-17483 (complement)	Fragment of maize chloroplast genome
13	10088-10275	188	99	PHI8999A	5333-5520 (complement)	Fragment of pat gene
14	10278-10358	81	100	NC_001666	137122-137202 (complement)	Fragment of maize chloroplast "ORF241"-hypothetical protein gene
15	10359-10612	254	N/A <sup>1</sup>	N/A	N/A	No significant homology detected
16	10613-11361	749	N/A <sup>1</sup>	N/A	N/A	No description available

<sup>1</sup>N/A; not applicable<sup>2</sup>LTR; long terminal repeat

## Example 6

## Confirmation of the Presence of Regions 1, 2, and 3 in an Unmodified Control Corn Line

PCR analysis was used to determine if Regions 1, 2, and 3 (Table 1) in the 5' flanking region of Event TC1507 are present in an unmodified control corn line used for transformation to produce maize event TC1507 and thus represents a border with corn genomic DNA. Nine different PCR analyses were carried out on genomic DNA prepared from TC1507 and the unmodified control corn line Hi-II (see Armstrong (1994) *The Maize Handbook*, ed. Freeling and Walbot, Springer-Verlag, New York, pp. 663-671, for information on Hi-II) as outlined in Table 2 using the primer sequences shown in Table 3. Two reactions were designed to amplify DNA within Region 1 of the 5' flanking region from by 25 to 324 (Reaction A—300 by amplicon); and from by 25 to 480 (Reaction B—456 by amplicon). The expected amplicons

45 were present in both the Hi-II unmodified corn line and in maize event TC1507. One PCR primer pair, Reaction C, spanned Region 2 to Region 3 of the 5' flanking region from by 759 to 1182 (424 by amplicon) and again produced PCR products of the expected size in both Hi-II and TC1507. Reaction D, spanned Region 1 to Region 3 of the 5' flanking region from by 415 to 1182 (768 by amplicon) and again produced PCR products of the expected size in both Hi-II and TC1507. Reactions E and F were designed as specific primer pairs for the pat gene region of the full-length insert of PHI8999A in TC1507 and thus an amplicon in the unmodified Hi-II corn line is not expected. The results indicate that both Reactions E and F are specific for a maize line transformed with a pat gene region and produce the expected amplicon, whereas no amplicon was produced in the unmodified Hi-II corn line. Reaction G was also designed as a primer pair that would produce an amplicon of 366 by in the maize event TC1507 and no amplicon in the unmodified Hi-II corn line.

Reactions H and I were designed as specific primer pairs for TC1507 that would span the end of the transgenic insert

## US 8,901,378 B2

27

into the 5' flanking region. In both Reactions H and I, the reverse primer was located in the ubiquitin promoter region of the full-length PHI8999A insert (Region 9 in Table 1) and the forward primer was located in Region 5, the rpoC2 gene fragment (see Table 1). Reaction H and Reaction I both produced an amplicon in maize line TC1507 and did not produce an amplicon in the unmodified control corn line. These results indicate that both Reactions H and I are specific for the TC1507 event.

The PCR results show that the undescribed sequence (Region 1) is present in the unmodified corn line Hi-II and that Regions 1, 2 and 3, are contiguous in the unmodified corn line Hi-II. The DNA sequences amplified in Reactions A, B, C, and D are not unique to the 5' flanking region of maize event TC1507 but are also present in the unmodified corn line Hi-II.

28

TABLE 3

PCR primers for sequence 5' to the PHI8999A insert in TC1507 and for regions within the full-length insert of PHI8999A in maize event TC1507

Reaction	Amplicon Size (bp)	Primer Pair	Primer Sequences 5' to 3'
A	300	SEQ ID NO: 10	CCCCCTACCCACCGACGT TTAT
		SEQ ID NO: 11	TTGATTGGCAGGTCCGTG GGTC
B	456	SEQ ID NO: 10	CCCCCTACCCACCGACGT TTAT

TABLE 2

PCR reactions for sequence 5' to the PHI8999A insert in maize event TC1507 and for regions within the full-length insert of PHI8999A in maize event TC1507

Reaction	PCR Amplicon Location	Amplicon Size (bp)	Region in TC1507 flanking sequence or PHI8999A insert	Amplicon present in Hi-II	Amplicon present in maize line TC1507
A	25-324 bp in TC1507 flanking sequence	300	Region 1	Yes	Yes
B	25-480 bp in TC1507 flanking sequence	456	Region 1	Yes	Yes
D	415-1182 bp in TC1507 5' flanking sequence	768	Region 1 to Region 3	Yes	Yes
E Not Unique to TC1507	4750-5794 bp in PHI8999A	1045	Region 9 (in full-length insert of PHI8999A 35S promoter to pat gene)	No	Yes
F Not Unique to TC1507	4827-5308 bp in PHI8999A	482	Region 9 (in full-length insert of PHI8999A 35S promoter to pat gene)	No	Yes
Detects cry1F fragment in 5' flanking region	cry1F sequence in 5' flanking sequence and in full-length insert of PHI8999A	366	Spans 335 bp cry1F sequence in 5' flanking sequence and same sequence in the full-length insert	No	Yes
H Unique to TC1507	2158 bp in Region 5 (rpoC2 gene fragment) to 3069 bp in Region 9 (full-length insert of PHI8999A)	912	Region 5 to Region 9 Unique to Insertion Event [SPANS UNIQUE JUNCTION REGIONS]	No	Yes
I Unique to TC1507	2158 bp in Region 5 (rpoC2 gene fragment) to 3001 bp in Region 9 (full-length insert of PHI8999A)	844	Region 5 to Region 9 Unique to Insertion Event [SPANS UNIQUE JUNCTION REGIONS]	No	Yes



US 8,901,378 B2

29

TABLE 3-continued

PCR primers for sequence 5' to the PHI8999A insert in TC1507 and for regions within the full-length insert of PHI8999A in maize event TC1507			
Reaction	Amplicon Size (bp)	Primer Pair	Primer Sequences 5' to 3'
C	424	SEQ ID NO: 12	CACAACGGGCACAGAAACA CGAA
		SEQ ID NO: 13	GCGCACCACCGGAACAA AATG
		SEQ ID NO: 14	TCCTCGCATTAAATGCTC CTGC
D	768	SEQ ID NO: 15	CCTGGCAGCATTTGACGC ATGT
		SEQ ID NO: 14	TCCTCGCATTAAATGCTC CTGC
E	1045	SEQ ID NO: 6	TAGAGGACCTAACAGAAC TCGCCGT
		SEQ ID NO: 7	GAGCTGGCAACTCAAAAT CCCTTT
F	482	SEQ ID NO: 8	AAAATCTTCGTCAACATG GTGGAGC
		SEQ ID NO: 9	TAATCTCAACTGGTCTCC TCTCCGG
G	366	SEQ ID NO: 19	GGCTCGGACTCGACCTTT CTAT
		SEQ ID NO: 20	GCAGTTCTTGAAGAATGA GTGA
H	912	SEQ ID NO: 1	GTAGTACTATAGATTATA TTATTCGTAGAG
		SEQ ID NO: 2	GCCATACAGAACTCAAAA TCTTTTCCGGAG
I	844	SEQ ID NO: 2	GCCATACAGAACTCAAAA TCTTTTCCGGAG
		SEQ ID NO: 23	CTTCAACAAGTGTGACA AA

## Example 7

## Flanking Sequence 3' to Inserted Transgenic DNA in Maize Event TC1507

Two separate PCR approaches were used to extend the length of the sequence information 3' to the full-length PHI8999A insert in maize event TC1507. In the first approach PCR primer pairs were designed to amplify a product that spanned the junction between the full-length insert and the inverted ORF25 terminator, see FIG. 1 for a depiction of the inverted ORF25 terminator. A forward primer was located at the end of the full-length PHI8999A insert and a series of reverse primers were located at 100 by intervals in the inverted sequence. In this manner the length of the inverted fragment present in the maize event TC1507 could be determined within a 100 by region based on the successful PCR reactions. This method indicated the inverted fragment contained the majority of the ORF25 terminator but no Cry1F sequence. PCR fragments were isolated and sequenced from this region.

In the second approach PCR primers were designed to walk out into the flanking DNA sequence from the inverted ORF25 terminator region as determined in the PCR experiment

30

described above. Genomic DNA isolated from two to three individual plants of event TC1507 and an unmodified control corn line was digested with various restriction enzymes and then ligated to adaptors specific for the restriction enzyme used for digestion (Universal Genome Walker™ Kit, Clontech Laboratories, Inc. and Devon et al. (1995) *Nucleic Acids Res.* 23:1644-1645). Primary PCR was carried out using an ORF25 terminator specific primer and a primer homologous to the adaptor sequence ligated onto the digested DNA. In order to increase the specificity of the reaction a nested secondary PCR was performed again with another ORF25 terminator specific primer and a secondary primer homologous to the adaptor sequence with the secondary primers being internal to the respective primers used in the primary PCR. Products produced by the nested PCR were analyzed by agarose gel electrophoresis and fragments unique to TC1507 DNA samples were isolated and sequenced. Fragments were amplified from both the ORF25 terminator contained within the full-length insert and from the targeted (inverted) ORF25 terminator on the 3' end of the full-length PHI8999A insert. Fragments from the full-length insert were of a predicted size based on the knowledge of the restriction enzyme sites located in the full-length insert. Fragments produced from the 3' inverted ORF25 terminator appeared as fragments of unexpected size. Sequence analysis of amplified fragments from the 3' inverted ORF25 terminator resulted in flanking DNA sequence of 1043 bp. Resultant sequence from the above series of genome walking experiments was used to design additional primers to walk further out from the insert into the bordering maize genome with a final 3' flanking sequence, of 2346 bp.

In order to describe the TC1507 3' flanking sequence, homology searching was done against the GenBank public databases using the Basic Local Alignment Search Tool (BLAST). The BLAST program performs sequence similarity searching and is particularly useful for identifying homologs to an unknown sequence. In addition to searching the public databases, alignments were performed using SeqMan 4.05™, Martinez and Needleman-Wunsch alignment algorithms (DNASTAR Inc.) to look for homology between the TC1507 3' flanking sequence and the PHI8999A transgenic insert. The results of these homology searches are presented in Table 1. The percent identity values indicate the percentage of identical matches across the length of the sequences analyzed. The results of similarity searching for the 3' flanking sequence indicate high homology with three regions of maize chloroplast DNA, a 188 by fragment of the pat gene, and 254 by of DNA (Region 15, Table 1) with no significant homology. An additional 749 by (Region 16) beyond Region 15 (see Table 1) was also sequenced. No similarity searching results are available for Region 16.

PCR analysis on control and TC1507 genomic DNA determined that the 254 by sequence (Region 15, fragment of maize chloroplast "ORF241") is present in the maize genome. The DNA sequence of Region 15 in the 3' flanking region is not unique to the 3' flanking region of maize event TC1507 but is also present in the unmodified control corn line. The TC1507 3' flanking sequence is presented in Example 8 and diagrammed in FIG. 1.

US 8,901,378 B2

31

Example 8

32

Sequence of the Region 3' to the End of the  
Full-Length Insert DNA in Maize Event TC1507

A description of each region is in Table 1.

Region 10 Fragment of ORF25 Terminator (complement)

(SEQ ID NO: 38)

9016 CTCAC TCCGCTTGAT CTTGGCAAAG ATATTGACG  
9051 CATTATTAG TATGTGTTAA TTTTCATTG CAGTGCAGTA TTTTCTATTC  
9101 GATCTTTATG TAATTCGTTA CAATTAATAA ATATTCAAAT CAGATTATTG  
9151 ACTGTCATTT GTATCAAATC GTGTTTAATG GATATTTTTA TTATAATATT  
9201 GATGATATCT CAATCAAAC GTAGATAATA ATAATATTTA TTAAATATTT  
9251 TTGCGTCGCA CAGTGAAAT CTATATGAGA TTACAAAATA CCGACAACAT  
9301 TATTTAAGAA ACATAGACAT TAACCCCTGAG ACTGTTGGAC ATCAACGGGT  
9351 AGATTCCCTC ATGCATAGCA CCTCATTCTT GGGGACAAAA GCACGGTTTG  
9401 GCCGTTCCAT TGCTGCACGA ACGAGCTTTG CTATATCCTC GGGTTGGATC  
9451 ATCTCATCAG GTCCAATCAA ATTTGTCCAA GAACTCATGT TAGTCGCAAC  
9501 GAAACCGGG CATATGTCGG GTATCTCGAG CTCGCGAAAG CTTGGCTGCA  
9551 GGTCGACGGA TCCTT

Region 11 Fragment of maize chloroplast rps12 rRNA gene (complement)

(SEQ ID NO: 39)

9566 CAACA AAAGGGTACC TGTACCCGAA ACCGACACAG  
9601 GTGGGTAGGT AGAGAATACC TAGGGGCGCG AGACAACTCT CTCTAAGGAA  
9651 CTCGGCAAAA TAGCCCCGTA ACTTCGGGAG AAGGGGTGCC CCC

Nucleotides 9694-9695 (CG) connect Region 11 to Region 12.

Region 12 Fragment of maize chloroplast genome

(SEQ ID NO: 40)

9696 CTAAC  
9701 AATAAACGAA TACGGTTTAT GTATGGATTC CCGTAAAATA CCGGTACTCG  
9751 ATTTCATAAG AGTCGAATAG GAAGTTAAGA TGAGGGTGGT ATCATCATAA  
9801 AAATGGAGTA GTATCCTAAA TTATACTAAT CCACGTATGA TATGTATGCC  
9851 TTTCTTATC AACCGGAAGT AGTGCAAAAA AAATTCTATA CTGCACTGCT  
9901 CTCTTTTAC TGAGAAATGC AAAAAATAA AAGTGAAGTA AGGGTGCCCC  
9951 ATAGATATTT GATCTTGCCT CCTGTCCCC CCCCCCTTT TTCAATCAAAA  
10001 ATTTCCATGA AAAAAGAAAA GATGAATTG TCCATTCAAT GAACCTAGT  
10051 TCGGGACTGA CGGGGCTCGA ACCCGCAGCT TCCGCCT

Region 13 Fragment of pat gene (complement)

(SEQ ID NO: 41)

10088 GTT CCTAGCCTTC  
10101 CAGGGCCCAG CGTAAGCAAT ACCAGCCACA GCACCTCAA CCTCAGCAAC  
10151 CAACCAAGGG TATCTATCTT GCAACCTCTC TAGATCATCA ATCCACTCTT  
10201 GTGGTGTGTTG TGGCTCTGTC CTAAAGTTCA CTGTAGACGT CTCAATGTAA  
10251 TGGTTAACGA TATCACAAC CGCGG

US 8,901,378 B2

33

34

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Nucleotides 10276-10277 (AA) connect Region 13 to Region 14.  
 Region 14 Fragment of maize chloroplast ORF241 (complement)  
 (SEQ ID NO: 42)

10278 CAC AAGAACGAAA GCACCTTTTC

10301 ATTCTTTCAT ATACTAGGGG TTTTACTTG GAAAAGACAA TGTTCATAC

10351 TAAAGGAT

Region 15 Maize genomic (no significant homology)  
 (SEQ ID NO: 43)

10359 AG CTGCAGAAGC CGCCACCGTC TTGAGGACCT TCCGGGGAGC

10401 CAGACCGGTC GAACCGTGCC TCCACTTGCT AAGGAGAAAG GGAAATCAG

10451 GGCCAGGACA TACGAAGGAG GAGCCAGAAC GAAGATATCC TAAGATACTT

10501 ACTCGCTCCG GGCCATGATC AATCATGCCT GTGGGGAGGT CTCTCGCACC

10551 TCATATCCATG AAGGTACCAC CGAGGTCTGC CCCGCCGCCG GCTTCGGTAC

10601 CGTCCTCGCC TT

Region 16 Maize genomic  
 (SEQ ID NO: 44)

10613 GGGCGCCC GAGGCACCCG GGGGATGGAC TGCCAGGCG

10651 CAGCCACGAC GACCCAAGGA TCACCTCCT GCGCAGTCGG CACGAGCAAT

10701 AGTTCTCGGG GAACAGGCAG CTTGGCCTGA CTCCTCGGG TCACCTCAAC

10751 TACCTCGGCC GAGGGGTCAA GTACCCCTC AGTCCGCCCC CGCTCTTCGG

10801 ACCGGGACCC CGACGTCCCG GCGCCGGATA CCGACGGCAC CAGCCCGCTC

10851 GGGGGCTGGC TTGACGACCC CTGGCCAGC CTCAGATCTG GGCTGAGGCC

10901 GAGGCAGGCG GCCATGTCGT CGTCTTCATC ATCGTCTTCA TCATCGTCGT

10951 CGTCATCAGG CGTCTCCGGC GACGGCTCCC TTGGGAGCCC CTCCTCTCC

11001 TGCCGACGAC GAAGCCTTTC CAAGGCATCC CGAGCCACG TCCGCTCGTG

11051 GGCCCGAGCC TTCTTTGCGT CCTTCTTCTC CTTCTCTTC TCCGCGGTGA

11101 CCCTCCGCGC AGCTCGGTCC ACCGCATCCT CCGGGACTGG TGGCAGGGAA

11151 GGCTTGTGAT GCCCTACCTC CTGGAGACAG ACGAAAAGTC TCAGCTATGA

11201 GAACCGAGGG CAATCTGACG CAAGAAGGAA GAAGGAGCGG ATACTACCA

11251 GAGACACGCA CCCGCGATCG GGACGCATTA AGGGCTGGGA AAAAGTGCCG

11301 GCCTCTAATT TCGCTACCGT GCCGTCCACC CACCTGTGGA GGTATCGAT

11351 GGAAGGGGA A

## Example 9

Confirmation of the Presence of Region 15 in the  
Unmodified Control Corn Line

PCR analysis was used to determine if the undescribed region of sequence on the end of the 3' flanking sequence (Region 15 in Table 1) is present in the unmodified control corn line used for transformation to produce maize event TC1507 and thus represents a border with corn genomic DNA. Successful PCR amplification of Region 15 in both maize line TC1507 and the unmodified Hi-II control corn line revealed that Region 15 was indeed present in corn genomic DNA. Five different PCR analyses were carried out on genomic DNA prepared from TC1507 and the unmodified Hi-II control corn line as outlined in Table 7 below using the primer sequences shown in Table 8. Three reactions were designed to amplify DNA within Region 15 of the 3' flanking region; Reaction L—producing a 175 by amplicon, Reaction

M—producing a 134 by amplicon, and Reaction N—producing a 107 by amplicon. The expected amplicons were present in both the unmodified control corn line and in maize line TC1507. Reactions J and K were designed as specific primer pairs for TC1507 that would span the end of the insert into the 3' flanking region. In Reaction J, the forward primer was located in the pat gene fragment on the 3' end of the full-length PHI8999A insert (Region 13 in Table 1) and the reverse primer was located in the undefined Region 15. In Reaction K the forward primer was located in the chloroplast hypothetical protein gene on the 3' end of the full-length insert (Region 14 in Table 1) and the reverse primer was located in the undefined Region 15. Both Reaction J and Reaction K produced an amplicon in maize line TC1507 and did not produce an amplicon in the unmodified control corn line. The results indicate that both Reactions J and K are specific for the TC1507 event.

The PCR results indicate that the undescribed sequence (Region 15) of the 3' flanking sequence of TC1507 is also

US 8,901,378 B2

35

present in genomic DNA isolated from the unmodified Hi-II control corn line. The DNA sequences amplified in Reactions L, M, and N are not unique to the 3' flanking region of TC1507 but are also present in the unmodified control corn line.

TABLE 7

PCR reactions for sequence 3' to the PHI8999A insert in maize event TC1507				
Reaction	Amplicon Size (bp)	Region in TC1507 3' flanking sequence	Amplicon present in Control	Amplicon present in maize line TC1507
J	342	Region 13 (pat gene fragment) to Region 15	No	Yes
K	252	Region 14 (chloroplast gene) to Region 15	No	Yes
L	175	Region 15	Yes	Yes
M	134	Region 15	Yes	Yes
N	107	Region 15	Yes	Yes

TABLE 8

PCR primers for sequence 3' to the PHI8999A insert in maize event TC1507				
Reaction	Amplicon Size (bp)	Primer Pair	Primer Sequences 5' to 3'	
J	342	SEQ ID NO: 3	TGTGGTGTGTTGTGGCTCTGTCTAA	
		SEQ ID NO: 5	GACCTCCCCACAGGCATGAT TGATC	
K	252	SEQ ID NO: 4	AGCACCTTTTCATTCTTTCA TATAC	
		SEQ ID NO: 5	GACCTCCCCACAGGCATGAT TGATC	
L	175	SEQ ID NO: 16	AAGCCGCCACCGTCTTGAGG ACCTT	
		SEQ ID NO: 5	GACCTCCCCACAGGCATGAT TGATC	
M	134	SEQ ID NO: 17	GTGGAACCGTGCTCCACTT GCTAA	
		SEQ ID NO: 5	GACCTCCCCACAGGCATGAT TGATC	
N	107	SEQ ID NO: 18	AGAAAGGGAAATCAGGGCC AGGAC	
		SEQ ID NO: 5	GACCTCCCCACAGGCATGAT TGATC	

## Example 10

## PCR Primers

DNA event specific primer pairs were used to produce an amplicon diagnostic for TC1507. These event primer pairs include, but are not limited to, SEQ ID NO: 1 and SEQ ID NO: 2; SEQ ID NO: 2 and SEQ ID NO: 23; SEQ ID NO: 3 and SEQ ID NO: 5; and SEQ ID NO: 4 and SEQ ID NO: 5. In addition to these primer pairs, any primer pair derived from SEQ ID NO: 26 and SEQ ID NO: 27 that when used in a DNA amplification reaction produces a DNA amplicon diagnostic for TC1507 is an aspect of the present invention. The amplification conditions for this analysis are illustrated in Table 9, however, any modification of these methods that use DNA

36

primers or complements thereof to produce an amplicon DNA molecule diagnostic for TC1507 is within the ordinary skill of the art. The preferred amplification conditions for reactions utilizing the PCR primers identified in SEQ ID NOS: 1, 2, and 23 are illustrated in Table 10. In addition, control primer pairs, which include SEQ ID NOS: 10 and 11; SEQ ID NOS: 10 and 12; SEQ ID NOS: 13 and 14; SEQ ID NOS: 14 and 15; SEQ ID NOS: 5 and 16; SEQ ID NOS: 5 and 17; and SEQ ID NOS: 5 and 18; for amplification of an endogenous corn gene are included as internal standards for the reaction conditions. Also included are primer pairs that will produce an amplicon in transgenic events containing a pat gene (SEQ ID NOS: 6 and 7; SEQ ID NOS: 8 and 9), and a primer pair that will produce an amplicon in transgenic events containing a cry1F gene (SEQ ID NOS: 19 and 20).

The analysis of plant tissue DNA extracts to test for the presence of the TC1507 event should include a positive tissue DNA extract control (a DNA sample known to contain the transgenic sequences). A successful amplification of the positive control demonstrates that the PCR was run under conditions which allow for the amplification of target sequences. A negative, or wild-type, DNA extract control in which the template DNA provided is either genomic DNA prepared from a non-transgenic plant, or is a non-TC1507 transgenic plant, should also be included. Additionally a negative control that contains no template corn DNA extract will be a useful gauge of the reagents and conditions used in the PCR protocol.

Additional DNA primer molecules of sufficient length can be selected from SEQ ID NO: 26 and SEQ ID NO: 27 by those skilled in the art of DNA amplification methods, and conditions optimized for the production of an amplicon that may differ from the methods shown in Table 9 or Table 10 but result in an amplicon diagnostic for event TC1507. The use of these DNA primer sequences with modifications to the methods shown in Table 9 and Table 10 are within the scope of the invention. The amplicon wherein at least one DNA primer molecule of sufficient length derived from SEQ ID NO: 26 and SEQ ID NO: 27 that is diagnostic for event TC1507 is an aspect of the invention. The amplicon wherein at least one DNA primer of sufficient length derived from any of the genetic elements of PHI8999A that is diagnostic for event TC1507 is an aspect of the invention. The assay for the TC1507 amplicon can be performed by using a Stratagene Robocycler, MJ Engine, Perkin-Elmer 9700, or Eppendorf Mastercycler Gradient thermocycler, or by methods and apparatus known to those skilled in the art.

TABLE 9

PCR Conditions:	
Conditions:	
Kit used: Perkin-Elmer AmpliTaq Gold kit	
Volume	Component
5 µl	template (10 ng/µl)
4 µl	2 µl each primer (10 µM)
2 µl	10X PCR Gold Buffer
2 µl	25 mM MgCl <sub>2</sub>
2 µl	50X dNTP's (10 mM)

US 8,901,378 B2

37

TABLE 9-continued

PCR Conditions: Conditions: Kit used: Perkin-Elmer AmpliTaq Gold kit	
Volume	Component
0.1 µl	Amplitaq Gold Polymerase
4.9 µl	H <sub>2</sub> O
20 µl	Total

Cycling Parameters  
GeneAmp ® PCR System 9700  
9 min 92° C.  
30 cycles:  
94° C. 30 sec  
60° C. 30 sec  
72° C. 1 min  
7 min 72° C.  
Hold 4° C.

TABLE 10

PCR Conditions used with the Advantage ®-GC 2 Polymerase Mix: Conditions: Kit used: Advantage ®-GC 2 Polymerase Mix	
Volume	Component
5 µl	template (10 ng/µl)
5 µl	2.5 µl each primer (10 µM)
10 µl	5x GC2 Buffer
10 µl	GC melt (1.0M final conc.)

38

TABLE 10-continued

PCR Conditions used with the Advantage ®-GC 2 Polymerase Mix: Conditions: Kit used: Advantage ®-GC 2 Polymerase Mix	
Volume	Component
1.5 µl	50X dNTP's (10 mM)
1.0 µl	Advantage GC2 Polymerase
17.5 µl	H <sub>2</sub> O
50 µl	Total

Cycling Parameters  
GeneAmp ® PCR System 9700  
5 min 94° C.  
35 cycles:  
94° C. 1 min  
60° C. 2 min  
72° C. 3 min  
7 min 72° C.  
Hold 4° C.

Having illustrated and described the principles of the present invention, it should be apparent to persons skilled in the art that the invention can be modified in arrangement and detail without departing from such principles. We claim all modifications that are within the spirit and scope of the appended claims.

All publications and published patent documents cited in this specification are incorporated herein by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

## SEQUENCE LISTING

&lt;160&gt; NUMBER OF SEQ ID NOS: 57

<210> SEQ ID NO 1  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Event specific primer sequence designed for TC1507

&lt;400&gt; SEQUENCE: 1

gtagtactat agattatatt attcgtagag

30

<210> SEQ ID NO 2  
<211> LENGTH: 30  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Event specific primer sequence designed for TC1507

&lt;400&gt; SEQUENCE: 2

gccatacaga actcaaaatc ttttccggag

30

<210> SEQ ID NO 3  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Event specific primer sequence designed for TC1507

&lt;400&gt; SEQUENCE: 3

US 8,901,378 B2

39

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tgtggtgttt gtggctctgt cctaa

25

<210> SEQ ID NO 4  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Event specific primer for TC1507

&lt;400&gt; SEQUENCE: 4

agcacctttt cattctttca tatac

25

<210> SEQ ID NO 5  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Genomic DNA primer sequence

&lt;400&gt; SEQUENCE: 5

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25

<210> SEQ ID NO 6  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer in full length insert, 35S promoter to  
pat gene

&lt;400&gt; SEQUENCE: 6

tagaggacct aacagaactc gccgt

25

<210> SEQ ID NO 7  
<211> LENGTH: 24  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer in full length insert, 35S promoter to  
pat gene

&lt;400&gt; SEQUENCE: 7

gagctggcaa ctcaaaatcc cttt

24

<210> SEQ ID NO 8  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer in full length insert, 35S promoter to  
pat gene

&lt;400&gt; SEQUENCE: 8

aaaatcttcg tcaacatggt ggagc

25

<210> SEQ ID NO 9  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer in full length insert, 35S promoter to  
pat gene

&lt;400&gt; SEQUENCE: 9

taatctcaac tggctcctc tccgg

25



US 8,901,378 B2

41

42

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<210> SEQ ID NO 10  
<211> LENGTH: 22  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

<400> SEQUENCE: 10

cccctacccc accgacgttt at 22

<210> SEQ ID NO 11  
<211> LENGTH: 22  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

<400> SEQUENCE: 11

ttgattggca ggtccgtggg tc 22

<210> SEQ ID NO 12  
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<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

<400> SEQUENCE: 12

cacaacggca cagaaacacg aa 22

<210> SEQ ID NO 13  
<211> LENGTH: 22  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

<400> SEQUENCE: 13

gcgcacccac cggaacaaaa tg 22

<210> SEQ ID NO 14  
<211> LENGTH: 22  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

<400> SEQUENCE: 14

tcctcgatt aaatgctcct gc 22

<210> SEQ ID NO 15  
<211> LENGTH: 22  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

<400> SEQUENCE: 15

cctggcacgc attgacgeat gt 22

<210> SEQ ID NO 16  
<211> LENGTH: 25  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

US 8,901,378 B2

43

44

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<400> SEQUENCE: 16

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25

<210> SEQ ID NO 17

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

<400> SEQUENCE: 17

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25

<210> SEQ ID NO 18

<211> LENGTH: 25

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Primer - Zea mays genomic DNA

<400> SEQUENCE: 18

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<210> SEQ ID NO 19

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Cry1F sequence primer

<400> SEQUENCE: 19

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<210> SEQ ID NO 20

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Cry1F sequence primer

<400> SEQUENCE: 20

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22

<210> SEQ ID NO 21

<211> LENGTH: 2829

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: 5' flanking sequence of event TC1507

<400> SEQUENCE: 21

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120

ggtaccgttt accagatttt tccagccgtt ttcggattta tcgggatata cagaaaacga

180

gacggaaacg gaataggttt ttttcgaaa acggtacggt aaacggtgag acaaacttac

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ccgacccacg gacctgcca tcaaccatca gccagtcagc ccatccccac agctatggcc

360

catggggcca tggtggccac atgccacgc aacgcaaggc agtaaggctg gcagcctggc

420

acgcattgac gcatgtggac acacacagcc gccgcctgtt cgtgtttctg tgccgttgtg

480

cgagactgtg actgcgagtg gcggagtcgg cgaacggcga ggcgtctccg gagtctggac

540

US 8,901,378 B2

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46

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aaattacca	2829

US 8,901,378 B2

47

48

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<210> SEQ ID NO 22  
<211> LENGTH: 2346  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: 3' flanking sequence of event TC1507  
  
<400> SEQUENCE: 22

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caaatcagat	tattgactgt	catttgtatc	aaatcgtgtt	taatggatat	ttttattata	180
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gacattaacc	ctgagactgt	tggacatcaa	cgggtagatt	ccttcacgca	tagcacctca	360
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US 8,901,378 B2

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agcggatact caccagagac acgcaccgcg gatcgggacg cattaagggc tgggaaaaag 2280
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ggggaa 2346
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<210> SEQ ID NO 23
<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Event specific primer sequence designed for
TC1507
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<400> SEQUENCE: 23
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<210> SEQ ID NO 24
<211> LENGTH: 11361
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: The sequence represents the transgenic insert
in maize line TC1507 as well as the sequence flanking
the insertion sites.
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<400> SEQUENCE: 24
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US 8,901,378 B2

51

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US 8,901,378 B2

53

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US 8,901,378 B2

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US 8,901,378 B2

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US 8,901,378 B2

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<210> SEQ ID NO 25
<211> LENGTH: 6186
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: The sequence represents the DNA molecule used
to transform maize line TC1507. This sequence
represents insert PHI8999A.
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<400> SEQUENCE: 25
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US 8,901,378 B2

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US 8,901,378 B2

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US 8,901,378 B2

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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Sequence that represents part of the PHI8999A  
insert as well as flanking sequence 5' to the  
insert.

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taacactcac tttgaggctc gggggctact gtcggggacc ataattaggg gtacccccaa 900  
gactccta atctagctggt aacccccatc agcacaaaag tgcaaaggcc tgatgggtgc 960  
gattaagtca aggtcggctc cactcaaggg acacgatctc gcctcgcccg agcccagcct 1020  
cgggcaaggg cggccgaccc cgaggattca cgtctcgccc gagggccccc tcaagcgacg 1080  
ggcacacctt cggctcgcgc gagggccatt ctctcgccgag aagcaacctt ggccagatcg 1140  
ccacaccgac cgaccgtatc gcaggagcat ttaatgcgag gatcgctga caccttatcc 1200  
tgacgcgcgc tcttcagtcg acagagccga agtgaccgca atcacttcgc cgctccactg 1260  
accgacctga caagaagaca gcgccgctg cgtcgctccg actgctgtgc cactcgacag 1320  
agtgaggctg acagcagcca agtcggcct cgggcgcat aggaagctcc gcctcgcccg 1380  
accctagggc tcggactcgg cctcggtcc ggaagacgac gaactacgct tcgcccagcc 1440  
ccagggcttg gactcagcct cggctccgga agacgacgaa ttccgctcg cccgacccca 1500  
gggctcggac tcggcctcgg ctccagaaga cgacgaactc cgctcgccc gacccaggg 1560  
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ctatgtactc ggtcttaggg gactggcctt tcaacaaact ggtacgaatc acaccgcac 1800  
attcaggaac tccgggacca ttgactctct agatgagata ccacctcaag acaacagcgg 1860  
cgacaccttg aatgactact cccatgtgct gaatcatgtt acctttgtgc gctggccagg 1920

US 8,901,378 B2

67

68

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tgagatctca ggttccgact catggagagc accaatgttc tcttggacgc atcgtagcgc	1980
tacccccaca aacaccattg atccagagag aatcactcat tcttcaagaa ctgcatactt	2040
tgccgagatc ctcaccccta aaggctactg acaatagtat tattggagtc gatacacaac	2100
tcacaaaaaa tacaagaagt cgactagggtg gattggtccg agtgaagaga aaaaaagcc	2160
atacagaact caaaatcttt tccggagata ttcatcttcc tgaagaggcg gataagatat	2220
taggtggcag tttgatacca ccagaaagag aaaaaaaga ttctaaggaa tcaaaaaaa	2280
ggaaaaattg ggtttatgtt caacggaaaa aatttctcaa aagcaaggaa agtattgtg	2340
gctatttato tatccgtgca gctgatatgg ccgcggtttg tgatatcgtt aaccattaca	2400
ttgagacgtc tacagtgaac tttaggacag agccacaaac accacaagag tggattgatg	2460
atctagagag gttgcaagat agataccctt ggttggttgc tgaggttgag ggtgttggtg	2520
ctggtattgc ttacgctggg ccctggaagg ctaggaacco tcaacctcag caaccaacca	2580
atggtatcta tcttgcaacc tctctagatc atcaatccac tcttggtgtg tttgtggctc	2640
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aagaggatc tcgaagcttc ggccggggcc catcgatato cgccggcatg cctgcagtgc	2760
agcgtgaccc ggtcgtgccc ctctctagag ataagtagca ttgcatgtct aagttataaa	2820
aaattaccac aactggaaga gcggttacc ccgacgaagc ttccggccggg gcccatcgat	2880
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gcattgcagt tctaagttat aaaaaattac cacatatctt ttttgtcaca cttgtttgaa	3000
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cgtccaccg ttggacttgc tccgctgtcg gcattccagaa attgcgtggc ggagcggcag	3660
acgtgagccg gcacggcagg cggcctctc ctctctcac ggcacggcag ctacggggga	3720
ttcttttccc accgctcctt cgctttccct tcctcgcccg ccgtaataaa tagacacccc	3780
ctccacaccc tctttcccca acctcgtgtt gttcggagcg cacacacaca	3830

&lt;210&gt; SEQ ID NO 27

&lt;211&gt; LENGTH: 3347

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: Sequence that represents part of the PHI8999A insert as well as flanking sequence 3' to the insert.

&lt;400&gt; SEQUENCE: 27

cccactatcc ttcgcaagac ccttcctcta tataaggaag ttcatttcat ttggagagga	60
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US 8,901,378 B2

69

70

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caggggtaccc	ggggatccac	catgtctccg	gagaggagac	cagttgagat	taggccagct	120
acagcagctg	atatggccgc	ggtttgtgat	atcgtaaac	attacattga	gacgtctaca	180
gtgaacttta	ggacagagcc	acaaacacca	caagagtgga	ttgatgatct	agagaggttg	240
caagatagat	acccttggtt	ggttgctgag	gttgaggggtg	ttgtggctgg	tattgcttac	300
gctgggccct	ggaaggctag	gaacgcttac	gattggacag	ttgagagtac	tgtttacgtg	360
tcacataggc	atcaaaggtt	gggcctagga	tccacattgt	acacacattt	gcttaagtct	420
atggaggcgc	aaggttttaa	gtctgtggtt	gctgttatag	gccttccaaa	cgatccatct	480
gttaggttgc	atgaggcttt	gggatacaca	gcccggggta	cattgcgcgc	agctggatac	540
aagcatggtg	gatggcatga	tgttggtttt	tggcaaaggg	attttgagtt	gccagctcct	600
ccaaggccag	ttaggccagt	taccagatc	tgagtcgacc	tgcaggcatg	cccgtgaaa	660
tcaccagtct	ctctctacaa	atctatctct	ctctataata	atgtgtgagt	agttcccaga	720
taaggaatt	agggttctta	tagggtttcg	ctcatgtgtt	gagcatataa	gaaaccctta	780
gtatgtattt	gtatttgtaa	aatacttcta	tcaataaaat	ttctaattcc	taaaaccaa	840
atccagtggc	gagctcgaat	tcgagctcga	gcccggttgg	atcctctaga	gtcgacctgc	900
agaagcttcg	gtccggcgcg	cctctagtgt	aagacacgtt	catgtcttca	tcgtaagaag	960
acactcagta	gtcttcggcc	agaatggcct	aactcaaggc	cctcactccg	cttgatcttg	1020
gcaaagatat	ttgacgcatt	tattagtatg	tgttaatttt	catttgagct	gcagtatttt	1080
ctattcgatc	tttatgtaat	tcgttacaat	taataaatat	tcaaatcaga	ttattgactg	1140
tcatttgtat	caaatcggtg	ttaatggata	tttttattat	aatattgatg	atatctcaat	1200
caaaacgtag	ataataataa	tattttattt	atatttttgc	gtcgcacagt	gaaaatctat	1260
atgagattac	aaaataccga	caacattatt	taagaaacat	agacattaac	cctgagactg	1320
ttggacatca	acgggttagat	tccttcatgc	atagcacctc	attcttgggg	acaaaagcac	1380
ggtttgccg	ttccattgct	gcacgaacga	gctttgctat	atcctcgggt	tgatcatct	1440
catcagggtc	aatcaaat	gtccaagaac	tcattgttagt	cgcaacgaaa	ccggggcata	1500
tgtcgggtat	ctcgagctcg	cgaaagcttg	gctgcaggtc	gacggatcct	tcaacaaaag	1560
ggtacctgta	cccgaaccg	acacaggtgg	gtaggtagag	aatacctagg	ggcgcgagac	1620
aactctctct	aagggaactcg	gcaaaatagc	cccgtaaact	cgggagaagg	ggtgcccccc	1680
gctaacaata	aacgaatacg	gtttatgtat	ggattccggg	aaaataccgg	tactcgattt	1740
cataagatgc	gaataggaag	ttaagatgag	ggtggtatca	tcataaaaa	ggagtagtat	1800
cctaaattat	actaatccac	gtatgatatg	tatgccttct	cttatcaacc	ggaagtagtg	1860
caaaaaaat	totatactgc	actgctctct	ttttactgag	aatgcaaaa	aaataaaagt	1920
gaagtaaggg	tgccccatag	atatttgatc	ttgectctcg	ccccccccc	ccttttttca	1980
tcaaaaaatt	ccatgaaaaa	agaaaagatg	aatttgtcca	ttcattgaac	cctagttcgg	2040
gactgacggg	gctcgaaccc	gcagcttcctg	cctgttecta	gccttcagg	gccagcgta	2100
agcaatacca	gccacagcac	cctcaacctc	agcaaccaac	caagggtatc	tatcttgcaa	2160
cctctctaga	tcacaaatcc	actcttggtg	tgtttggtgc	tctgtcctaa	agttcactgt	2220
agacgtctca	atgtaatggt	taacgatatc	acaaaccgcg	gaacacaaga	acgaaagcac	2280
cttttcattc	tttcatatac	taggggtttt	tacttggaag	agacaatgtt	ccatactaaa	2340
ggatagctgc	agaagccgcc	accgtcttga	ggaccttccg	gggagccaga	ccggtcgaac	2400
cgtgcctcca	cttgctaagg	agaaaggga	aatcagggcc	aggacatacg	aaggaggagc	2460

US 8,901,378 B2

71

72

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```
cagaacgaag atatcctaag atacttactc gctccggggc atgatcaatc atgcctgtgg 2520
ggaggtctct cgcacctcga tccatgaagg taccaccgag gtctgccccg ccgccggctt 2580
cggtagcgtc ctgccttgg ggcgccgagg cccccggggg atggactgcc caggcgcagc 2640
cacgacgacc caaggatcac cctcctgcgc agtcggcacg agcaatagtt ctcggggaac 2700
aggcagcttg gctgactcc ccgggggtcac ctcaactacc tcggccgagg ggtcaagtac 2760
cccctcagtc cgcctccgct cttcggaccg ggaccccgac gtcccgggcc cggataccga 2820
cggcaccagc ccgctcgggg gctggttga cgacccctgg ccagcctca gatctgggct 2880
gaggccgagg caggcggcca tgcgtcgtc ttcacatcgt tcttcacatc cgtcgtcgtc 2940
atcaggcgtc tccggcgacg gctcccttgg gagccctcc ctctcctgcc gacgacgaag 3000
cttttccaag gcaccccgag ccacagtcgc ctggtggggc cgagcctctt ttgcgtcctt 3060
cttctccttc ctcttctccg cggtgaccct ccgcgcagct cggccaccgc catcctccgg 3120
gactggtggc aggggaaggct tgtgatgcc tacctcctgg agacagacga aaagtctcag 3180
ctatgagaac cgagggcaat ctgacgcaag aagggaagaag gagcggatac tcaccagaga 3240
cacgcacccg cgatcgggac gcattaaggg ctgggaaaaa gtgccggcct ctaatttcgc 3300
taccgtgccg tccaccacc tgtggaggtc atcgatggga aggggaa 3347
```

```
<210> SEQ ID NO 28
<211> LENGTH: 669
<212> TYPE: DNA
<213> ORGANISM: Zea mays
```

```
<400> SEQUENCE: 28
```

```
actagtttcc tagcccgct cgtgcccta cccaccgac gtttatggaa ggtgccattc 60
cacggttctt cgtggccgcc cctaaggatg taaatggctg gtaaaatccg gtaaatcttc 120
ggtaccgttt accagatttt tccagcgtt ttcggattta tcgggatata cagaaaacga 180
gacgaaaacg gaataggttt ttttctgaaa acggtacggt aaacggtgag acaaacttac 240
cgtccgtttt cgtatttctc gggaaactct ggtatatctc cgtatttgc ccgtatttct 300
ccgacccacg gacctgcaa tcaacatca gccagtcagc ccatccccc acgtatggcc 360
catggggcca tgttggccac atgccacgc aacgcaaggc agtaaggctg gcagcctggc 420
acgcattgac gcattgtgac acacacagcc gccgcctgtt cgtgtttctg tgccgttggtg 480
cgagactgtg actgcgagt ggcgagtcgg cgaacggcga ggcgtctccg gagtctggac 540
tgccgctgtg gacagcgacg ctgtgacggc gactcggcga agccccaagc taccaagccc 600
ccaagtcccc atccatctct gcttctctgg tcatctcctt cccctggctg atctgcaggc 660
gccagaccg 669
```

```
<210> SEQ ID NO 29
<211> LENGTH: 200
<212> TYPE: DNA
<213> ORGANISM: Zea mays
```

```
<400> SEQUENCE: 29
```

```
gccgaagcat cacgaaacgc actaagacct cgaaggagtc aaaccactcc tccgaggcct 60
cgggggctac acccggcggg tgcgctcgcg cgcaccacac ggaacaaaat gtaaccgaga 120
aaggtcggto cccttgcaaa aaaagtgcga caaaagcctc caagcgagta ttaacactca 180
ctttgaggct cgggggctac 200
```

US 8,901,378 B2

73

74

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```
<210> SEQ ID NO 30
<211> LENGTH: 812
<212> TYPE: DNA
<213> ORGANISM: Zea mays
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (0)...(0)
<223> OTHER INFORMATION: Fragment of maize Huck-1 retrotransposon

<400> SEQUENCE: 30

tgtcggggac cataattagg ggtaccccca agactcctaa tctcagctgg taacccccat    60
cagcacaaag ctgcaaaggc ctgatgggtg cgattaagtc aaggctcggt cactcaagg    120
gacacgatct cgctcgcgcc gagcccagcc tcgggcaagg gcggccgacc cggaggattc    180
acgtctcgcc cgaggggccc ctcaagcgac gggcacacct tcggctcgcc cgaggcccat    240
tcttcgccga gaagcaacct tggccagatc gccacaccga ccgaccgtat cgcaggagca    300
tttaatgcga ggatcgctg acaccttata ctgacgcgcg ctcttcagtc gacagagccg    360
aagtgaccgc aatcacttcg ccgctccact gaccgacctg acaagaagac agcgccgctt    420
gcgtcgctcc gactgctgtg cactcgaca gactgaggct gacagcagcc aagtccggcc    480
tcgggcgcga taggaagetc cgctcgcgcc gacctagggt ctcgactcg gcctcggtc    540
cggaagacga cgaactacgc ttcgcccagc cccagggtt ggactcagcc tcggctccgg    600
aagacgacga attccgcctc gcccgacccc agggctcgga tcggcctcg gctccagaag    660
acgacgaact ccgctcgccc cgaccccagg gctcgactc agcctcggtt ccgaagacg    720
acgaactccg cctcgcccga ccccgagggt cggactcagc ctcggcctca gacgatggtc    780
tcgcctcgc ccgacccggg gctcgactc ga                                812
```

```
<210> SEQ ID NO 31
<211> LENGTH: 335
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Represents part of PHI8999A insert sequence -
                        fragment of Cry1F gene
```

```
<400> SEQUENCE: 31

cctttctatc ggacctgtc agatcctgtc ttctccgag gaggtttgg caatcctcac    60
tatgtactcg gtcttagggg agtggccttt caacaaactg gtacgaatca caccgcaca    120
ttcaggaact ccgggacctt tgactctcta gatgagatac cacctcaaga caacagcggc    180
gcaccttga atgactactc ccattgtgtg aatcatgtta cctttgtgct ctggccagggt    240
gagatctcag gttccgactc atggagagca ccaatgttct cttggacgca tcgtagcgct    300
acccccacaa acaccattga tccagagaga atcac                                335
```

```
<210> SEQ ID NO 32
<211> LENGTH: 321
<212> TYPE: DNA
<213> ORGANISM: Zea mays
<220> FEATURE:
<221> NAME/KEY: misc_feature
<222> LOCATION: (0)...(0)
<223> OTHER INFORMATION: Fragment of maize chloroplast rpoC2 gene
```

```
<400> SEQUENCE: 32

tcattcttca agaactgcat atcttgccga gatcctcaco cctaaaggta cttgacaata    60
gtattattgg agtcgataca caactcacia aaaatacaag aagtcgacta ggtggattgg    120
```

US 8,901,378 B2

75

76

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tccgagtgaa	gagaaaaaaa	agccatacac	aactcaaaat	cttttcgga	gatattcatt	180
ttctgaaga	ggcggataag	atattagggt	gcagtttgat	accaccagaa	agagaaaaaa	240
aagattctaa	ggaatcaaaa	aaaaggaaaa	attgggttta	tgttcaacgg	aaaaaatctc	300
tcaaaaacaa	ggaaaaactat	t				321

```
<210> SEQ ID NO 33
<211> LENGTH: 17
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Represents part of PHI8999A insert
sequence-fragment of ubiZM1(2) promoter; also a
fragment of the maize chloroplast trnI gene
```

<400> SEQUENCE: 33

gtggctattt atctatc 17

```
<210> SEQ ID NO 34
<211> LENGTH: 201
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Represents part of PHI8999A insert
sequence-fragment of pat gene
```

<400> SEQUENCE: 34

gcagctgata tggccgcggt ttgtgatatc gttaaccatt acattgagac gtctacagtg	60
aacttttagga cagagccaca aacaccacaa gagtggattg atgatctaga gaggttgcaa	120
gatagatacc ctgtggttgg ttgctgaggtt gagggtgttg tggtctggtat tgettacgct	180
gggccctgga aggctaggaa c	201

```
<210> SEQ ID NO 35
<211> LENGTH: 138
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Represents part of PHI8999A insert
sequence-fragment of pat gene (complement)
```

<400> SEQUENCE: 35

```
cctcaacctc agcaaccaac caatggtatc tatcttgcaa cctctctaga tcatcaatcc      60
actcttgtag tgtttgtggc tctgtcttaa agttcactgt agacgtctca atgtaaatgg      120
taacgatatc acaaaccg                                     138
```

```
<210> SEQ ID NO 36
<211> LENGTH: 15
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Represents part of PHI8999A insert
sequence-fragment of crylF gene (complement)
```

<400> SEQUENCE: 36

aqagaagagg gatct 15

```
<210> SEQ ID NO 37
<211> LENGTH: 118
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Represents part of PHI8999A insert
sequence-fragment of polylinker
```



US 8,901,378 B2

77

78

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&lt;400&gt; SEQUENCE: 37

```
cgaagcttcg gccggggccc atcgatatcc gcgggcatgc ctgcagtgea gcgtgacccg      60
gtcgtgcccc tctctagaga taatgagcat tgcattgtcta agttataaaa aattacca      118
```

&lt;210&gt; SEQ ID NO 38

&lt;211&gt; LENGTH: 550

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Artificial Sequence

&lt;220&gt; FEATURE:

<223> OTHER INFORMATION: Represents part of PHI8999A insert  
sequence-fragment of ORF25 terminator (complement)

&lt;400&gt; SEQUENCE: 38

```
ctcactccgc ttgatcttgg caaagatatt tgacgcattt attagtatgt gttaattttc      60
atttgcagtg cagtattttc tattcgatct ttatgtaatt cgttacaatt aataaatatt      120
caaatcagat tattgactgt catttgtatc aaatcgtgtt taatggatat ttttattata      180
atattgatga tatctcaatc aaaacgtaga taataataat atttatttaa ttttttgcg      240
tcgcacagtg aaaatctata tgagattaca aaataccgac aacattattt aagaacata      300
gacattaacc ctgagactgt tggacatcaa cgggtagatt ctttcattgca tagcacctca      360
ttcttgggga caaaagcacg gtttggccgt tccattgctg cacgaacgag ctttgctata      420
tcctcggggt ggatcatctc atcagggtcca atcaaatctg tccaagaact catgttagtc      480
gcaacgaaac cggggcatat gtcgggtatc tcgagctcgc gaaagcttgg ctgcagggtcg      540
acggatcctt                                     550
```

&lt;210&gt; SEQ ID NO 39

&lt;211&gt; LENGTH: 128

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Zea mays

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: misc\_feature

&lt;222&gt; LOCATION: (0)...(0)

<223> OTHER INFORMATION: Fragment of maize chloroplast rps12 rRNA gene  
(complement)

&lt;400&gt; SEQUENCE: 39

```
caacaaaagg gtacctgtac ccgaaaccga cacaggtggg taggtagaga atacctaggg      60
gcgcgagaca actctctcta aggaactcgg caaaatagcc ccgtaacttc gggagaaggg      120
gtgcccccc                                     128
```

&lt;210&gt; SEQ ID NO 40

&lt;211&gt; LENGTH: 392

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Zea mays

&lt;220&gt; FEATURE:

&lt;221&gt; NAME/KEY: misc\_feature

&lt;222&gt; LOCATION: (0)...(0)

&lt;223&gt; OTHER INFORMATION: Fragment of maize chloroplast genome

&lt;400&gt; SEQUENCE: 40

```
ctaacaataa acgaatacgg tttatgtatg gattccggta aaataccggt actcgatttc      60
ataagagtcg aataggaagt taagatgagg gtggtatcat cataaaaatg gagtagtata      120
ctaaattata ctaatccacg tatgatattg atgcctttcc ttatcaaccg gaagtagtgc      180
aaaaaaaatt ctatactgca ctgctctctt ttactgaga aatgcaaaaa aataaaagtg      240
aagtaagggt gccccataga tatttgatct tgccctcctg cccccccccc cttttttcat      300
```

US 8,901,378 B2

79

80

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caaaaatttc catgaaaaaa gaaaagatga atttgatcat tcattgaacc ctagtccggg	360
actgacgggg ctcgaaccgc cagcttcgc ct	392

<210> SEQ ID NO 41  
<211> LENGTH: 188  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Represents part of PHI8999A insert  
sequence-fragment of pat gene (complement)

&lt;400&gt; SEQUENCE: 41

gttcctagcc ttccagggcc cagcgtaagc aataccagcc acagcaccct caacctcagc	60
aaccaaccaa gggatatctat cttgcaacct ctctagatca tcaatccact cttgtgggtg	120
ttgtggctct gtcctaaagt tcaactgtaga cgtctcaatg taatgggttaa cgatatcaca	180
aaccgcgg	188

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<212> TYPE: DNA  
<213> ORGANISM: Zea mays  
<220> FEATURE:  
<221> NAME/KEY: misc\_feature  
<222> LOCATION: (0)...(0)  
<223> OTHER INFORMATION: Fragment of maize chloroplast ORF241  
(complement)

&lt;400&gt; SEQUENCE: 42

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caatgttcca tactaaagga t	81

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<212> TYPE: DNA  
<213> ORGANISM: Zea mays

&lt;400&gt; SEQUENCE: 43

agctgcagaa gccgccaccg tcttgaggac cttccgggga gccagaccgg tcgaaccgtg	60
cctccacttg ctaaggagaa agggaaaatc agggccagga catacgaagg aggagccaga	120
acgaagatat cctaagatac ttactcgctc cgggccatga tcaatcatgc ctgtggggag	180
gtctctcgca cctcgatcca tgaaggtacc accgaggtct gcccgcgcgc cggcttcggt	240
accgtcctcg cctt	254

<210> SEQ ID NO 44  
<211> LENGTH: 749  
<212> TYPE: DNA  
<213> ORGANISM: Zea mays

&lt;400&gt; SEQUENCE: 44

gggcgcccga ggcacccggg ggatggactg cccaggcgca gccacgacga cccaaggatc	60
accctcctgc gcagtcggca cgagcaatag ttctcgggga acaggcagct tggcctgact	120
ccccggggto acctcaacta cctcggcgga ggggtcaagt accccctcag tccgcccccg	180
ctcttcggac cgggaccccg acgtcccggc cccggatacc gacggcacca gcccgctcgg	240
gggctggctt gacgacctt ggcccagcct cagatctggg ctgaggcgga ggcaggcggc	300
catgtcgtcg tcttcatcat cgtcttcac atcgtcgtcg tcatcaggcg tctccggcga	360
cggtccctt gggagccctt cctctcctg ccgacgacga agcctttcca aggcaccccg	420

US 8,901,378 B2

81

82

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agcccacgtc cgctcgtggg cccgagcctt ctttgcgtcc ttctttctct tcctttcttc 480
cgcggtgacc ctccgcgcag ctccgtccac cgcctcctcc gggactggtg gcaggggaagg 540
cttgtgatgc cctacctctt ggagacagac gaaaagtctc agctatgaga accgagggca 600
atctgacgca agaaggaaga aggagcggat actcaccaga gacacgcacc cgcgatcggg 660
acgcattaag ggctgggaaa aagtgccggc ctctaatttc gctaccgtgc cgtccacca 720
cctgtggagg tcctcgatgg gaaggggaa 749
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<400> SEQUENCE: 45
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```
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<211> LENGTH: 20
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<220> FEATURE:
<223> OTHER INFORMATION: Biolistic transformation event 5' flanking
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<220> FEATURE:
<223> OTHER INFORMATION: Biolistic transformation event 5' flanking
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<212> TYPE: DNA
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<220> FEATURE:
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```
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```
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```

```
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<220> FEATURE:
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```

```
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```
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```

US 8,901,378 B2

83

84

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<210> SEQ ID NO 50  
<211> LENGTH: 20  
<212> TYPE: DNA  
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<220> FEATURE:  
<223> OTHER INFORMATION: Biolistic transformation event 5' flanking  
sequence; junction between regions 7b and 7c  
  
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tcacaaaccg agagaagagg 20  
  
<210> SEQ ID NO 51  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Biolistic transformation event 5' flanking  
sequence; junction between regions 7c and 8  
  
<400> SEQUENCE: 51  
  
agagggatct cgaagcttcg 20  
  
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<211> LENGTH: 20  
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<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Biolistic transformation event 5' flanking  
sequence; junction between regions 8 and 9  
  
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aaaattacca caactggaag 20  
  
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<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Biolistic transformation event 3' flanking  
sequence; junction between regions 9 and 10  
  
<400> SEQUENCE: 53  
  
agctatgttt ctactccgc 20  
  
<210> SEQ ID NO 54  
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<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Biolistic transformation event 3' flanking  
sequence; junction between regions 10 and 11  
  
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<210> SEQ ID NO 55  
<211> LENGTH: 20  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Biolistic transformation event 3' flanking  
sequence; junction between regions 11 and 12  
  
<400> SEQUENCE: 55  
  
gtgcccccg ctaacaataa 20  
  
<210> SEQ ID NO 56

US 8,901,378 B2

85

86

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<211> LENGTH: 20
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Biolistic transformation event 3' flanking
sequence; junction between regions 12 and 13

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<400> SEQUENCE: 56

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```

20

```

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<211> LENGTH: 20
<212> TYPE: DNA
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<220> FEATURE:
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sequence; junction between regions 13 and 14

```

```

<400> SEQUENCE: 57

```

```

aaaccgcgga acacaagaac

```

20

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What is claimed is:

1. A corn plant comprising a DNA construct, said DNA construct comprising a first and a second expression cassette, wherein said first expression cassette in operable linkage comprises

- (a) a maize ubiquitin promoter;
- (b) a 5' untranslated exon of a maize ubiquitin gene;
- (c) a maize ubiquitin intron;
- (d) a Cry1F encoding DNA molecule; and
- (e) a 3' ORF25 transcriptional terminator; and said second expression cassette comprising in operable linkage
  - (i) a CaMV 35S promoter;
  - (ii) a pat encoding DNA molecule; and
  - (iii) a 3' transcriptional terminator from (CaMV) 35S comprising the nucleic acid sequence of positions 5843 to 6032 of SEQ ID NO: 25;
 and wherein the first and second expression cassettes are flanked by SEQ ID NO: 26 at the 5' end and SEQ ID NO: 27 at the 3' end.

2. The corn plant of claim 1, wherein the corn plant is a seed.

3. A method of producing an insect resistant corn plant comprising:

- (a) breeding with an insect resistant corn plant comprising in its genome DNA the DNA construct of claim 1 and
- (b) selecting a progeny of (a) by analyzing the genome of the progeny for the presence of the DNA construct of claim 1.

4. The method of claim 3, wherein breeding comprises crossing the insect resistant corn plant of (a) with a second plant.

5. The method of claim 3, wherein the insect resistant corn plant of (a) is the pollen parent for the crossing.

6. The method of claim 4, wherein the second plant is the pollen parent for the crossing.

7. The method of claim 3, wherein the progeny comprises the insect resistance of the insect resistant corn plant of (a).

8. The corn plant of claim 1, wherein the DNA construct comprises the polynucleotide of SEQ ID NO: 24.

\* \* \* \* \*

# **Exhibit H**





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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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12/970,052

12/16/2010

Scott Diehn

3700

5362

27310 7590 02/04/2013  
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P.O. BOX 552  
JOHNSTON, IA 50131-0552

EXAMINER

BOLLAND, JEFFREY R

ART UNIT

PAPER NUMBER

1638

NOTIFICATION DATE

DELIVERY MODE

02/04/2013

ELECTRONIC

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

toni.farris@pioneer.com  
michelle.rees@pioneer.com  
sue.smith@pioneer.com

# 7462

**Office Action Summary****Application No.**

12/970,052

**Applicant(s)**

DIEHN ET AL.

**Examiner**

JEFFREY BOLLAND

**Art Unit**

1638

**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --****Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

- 1) ☒ Responsive to communication(s) filed on 12 October, 2012.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on \_\_\_\_; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims**

- 5) ☒ Claim(s) 1-44 is/are pending in the application.
- 5a) Of the above claim(s) 33-44 is/are withdrawn from consideration.
- 6) ☒ Claim(s) 15 is/are allowed.
- 7) ☒ Claim(s) 1-14 and 16-32 is/are rejected.
- 8) ☐ Claim(s) \_\_\_\_ is/are objected to.
- 9) ☐ Claim(s) \_\_\_\_ are subject to restriction and/or election requirement.

\* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see [http://www.uspto.gov/patents/init\\_events/pph/index.jsp](http://www.uspto.gov/patents/init_events/pph/index.jsp) or send an inquiry to [PPHfeedback@uspto.gov](mailto:PPHfeedback@uspto.gov).

**Application Papers**

- 10) ☐ The specification is objected to by the Examiner.
- 11) ☒ The drawing(s) filed on 16 December, 2010 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.
- Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).
- Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some \* c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☒ Information Disclosure Statement(s) (PTO/SB/08)  
Paper No(s)/Mail Date 21 March, 2011 and 25 March, 2011.
- 3) ☐ Interview Summary (PTO-413)  
Paper No(s)/Mail Date. \_\_\_\_.
- 4) ☐ Other: \_\_\_\_.

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 2

### **DETAILED ACTION**

Claims 1-44 are pending, only claims 1-32 are considered as claims 33-44 are withdrawn from consideration

### ***Election/Restrictions***

Applicant's election of Group I (Claims 1-32) without traverse made on 12 October, 2012 is acknowledged.

### ***Claim Objections***

1. **Claim 1** may benefit from the addition of a colon at the end of the lines that begin with "said third expression cassette" and "said fourth expression cassette".

### ***Claim Rejections - 35 USC § 112***

The following is a quotation of 35 U.S.C. 112(b):

(B) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

2. **Claims 5-7, 16-23, 27, and 29-30** are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention. All dependent claims are included in this rejection.

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 3

3. **Claim 5** is indefinite as the event name is an arbitrary designation in the absence of a deposit organization and accession number such as American Type Culture Collection (ATCC) with Accession No. PTA-004114-3.
4. **Claims 16 and 20** are indefinite in the recitation of "...a nucleotide sequence which is or is complementary to a sequence selected from the group consisting of...". It is unclear if Applicant is claiming the sequences in either orientation or if words are missing from the claim. For purposes of examination, it was assumed sequences are claimed in either orientation. Such treatment does not relieve Applicant of the responsibility to respond to this rejection.
5. **Claims 27 and 29-30** are indefinite in the recitation of "...event DP-004114-3...". The event name is an arbitrary designation in an independent claim in the absence of a deposit organization and accession number such as American Type Culture Collection (ATCC) with Accession No. PTA-004114-3.
6. **Claim 29** recites the limitation "the step" in line 1. There is insufficient antecedent basis for this limitation in the claim.

The following is a quotation of 35 U.S.C. 112(a):

(a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 4

### Scope of Enablement

XX. **Claims 5-13 and 16-32** are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The specification fails to provide enablement for the genotype of the corn even DP-004114-3 wherein the genotype comprises the four expression cassettes flanked by SEQ ID NO:27 and 28. Furthermore, the specification fails to enable the genotype of the corn event DP-004114-3 wherein the genotype comprises SEQ ID NO:27 and 28 without the four expression cassettes in between.

### *SEQ ID NO:27 and 28 Do Not Encode a Pest Resistance Protein*

Claims 24-26 are drawn to a method of producing hybrid corn seed comprising SEQ ID NO:27-28. The basis for the instant application appears drawn to a corn plant, in one instance a method of producing hybrid corn seed, comprising a DNA construct comprising four operably expression cassettes that encode three Cry toxins and a gene that confers tolerance to phosphinothricin. The sequences of SEQ ID NO:27 and 28 do not encode any of the Cry proteins or the phosphinothricin resistance gene. Applicant has failed to provide any guidance on a plant that comprises SEQ ID NO:27 and 28 but

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 5

not the DNA construct comprising the four expression cassettes. Applicant has further failed to provide any guidance on the method of producing hybrid corn seed comprising SEQ ID NO:27 and 28 but not the DNA construct comprising the four expression cassettes. It is not apparent what one would do with a seed that is phenotypically no different than wild-type. Applicant has also failed to teach how to use such a plant or seed.

The specification fails to provide guidance on this matter and further fails to provide any working examples that demonstrate the use of a plant comprising SEQ ID NO:27-28 without the DNA construct comprising the four expression cassettes.

*Deposit Requirement Has Not Been Met*

The claims are directed to specific seed. Since the seed are essential to the claimed invention, they must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the seed are not so obtainable or available, a deposit of said corn seed comprising event DP-004114-3 may satisfy the requirements of 35 U.S.C. 112. The specification discloses the deposit of corn seed with the American Type Culture Collection, ATCC Deposit Number PTA-11506, however no statement has been provided stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent (para spanning pg 10-11). A deposit is required for enablement purposes.

If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicants, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction



Application/Control Number: 12/970,052

Page 6

Art Unit: 1638

released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

- (a) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;
- (b) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent;
- (c) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the enforceable life of the patent, whichever is longer;
- (d) a test of the viability of the biological material at the time of deposit (see 37 CFR 1.807); and,
- (e) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CFR 1.809(d) should be added to the specification. See 37 CFR 1.801 - 1.809 [MPEP 2401-2411.05] for additional explanation of these requirements.

### ***Claim Rejections - 35 USC § 103***

The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

This application currently names joint inventors. In considering patentability of the claims under 35 U.S.C. 103(a), the examiner presumes that the subject matter of

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 7

the various claims was commonly owned at the time any inventions covered therein were made absent any evidence to the contrary. Applicant is advised of the obligation under 37 CFR 1.56 to point out the inventor and invention dates of each claim that was not commonly owned at the time a later invention was made in order for the examiner to consider the applicability of 35 U.S.C. 103(c) and potential 35 U.S.C. 102(e), (f) or (g) prior art under 35 U.S.C. 103(a).

10. **Claims 1-4 and 14** are rejected under 35 U.S.C. 103(a) as being unpatentable over Bing et al (U.S. PG PUB 2008/0178357 A1) in view of Barbour et al (WO 2004099447 A2), further in view of each of Castle et al (2006. Curr Opin Biotechnol. 17:105-112), Hua et al (2001. Appl Environ Microbiol. 67(2):872-879), and Kaiser-Alexnat et al (2009. Insect Path Insect Parasit Nematodes. 45:235-238).

The claims are drawn to a DNA construct comprising four expression cassettes in operable linkage, wherein a first cassette comprises a maize ubiquitin promoter, a 5' untranslated exon of a maize ubiquitin gene, a maize ubiquitin first intron; a Cry1F encoding DNA molecule, and a poly(A) addition signal from ORF 25 terminator; a second cassette comprises a maize ubiquitin promoter, a 5' untranslated exon of a maize ubiquitin gene, a maize ubiquitin first intron, a Cry34Ab1 encoding DNA molecule, and a PinII transcriptional terminator; a third cassette comprises a wheat peroxidase promoter, a Cry35Ab1 encoding DNA molecule, and a PinII transcriptional terminator; and a fourth cassette comprises a CaMV 35S promoter, a *pat* encoding DNA molecule, and a 3' transcriptional terminator from CaMV 35S; and a plant comprising the construct wherein the plant may be corn.

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 8

Bing et al teach a DNA construct comprising three expression cassettes in operable linkage, the first cassette comprising a maize ubiquitin promoter, a 5' untranslated exon 1 of a maize ubiquitin gene, a maize ubiquitin first intron, a Cry34Ab1 encoding DNA molecule, and a PinII transcriptional terminator; the second cassette comprising a wheat peroxidase promoter, a Cry35Ab1 encoding DNA molecule, and a PinII transcriptional terminator; and the third cassette comprising a CaMV 35S promoter, a PAT encoding DNA molecule, and the 3' CaMV 35S transcriptional terminator (pg 41, Claim 1). Bing et al also teach said DNA construct in a plant, wherein the plant may be a corn plant (pg 41, Claims 2-3).

Bing et al do not teach a fourth cassette comprising a Cry1F encoding DNA molecule and the associated regulatory sequences.

Barbour et al teach a DNA construct comprising a maize ubiquitin promoter, a 5' untranslated exon of a maize ubiquitin gene, a maize ubiquitin first intron, a Cry1F toxin encoding DNA molecule, and a poly(a) terminator from ORF25 (pg 3, ln 2-15).

At the time the invention was made, it would have been obvious to one of ordinary skill in the art to use common techniques known in the art to combine the DNA constructs of Bing et al and Barbour et al, making obvious the DNA construct comprising the four expression cassettes of the instant claims. One of ordinary skill in the art would have been motivated to do so because Castle et al teach that stacked genes that increase the spectrum of target insects contributes to improved insect resistance management (pg 109, para spanning left and right columns). It is well known

Application/Control Number: 12/970,052

Page 9

Art Unit: 1638

in the art that Cry1F is toxic to Lepidopterans and the Cry34Ab1/Cry35Ab1 binary system is toxic to Coleopterans.

One would be motivated to combine the teachings of Bing et al with Barbour et al as Hua et al teach that Cry1F binds to at least two ligands in the gut of the Eastern cornborer with molecular weights of 154 kDa and 220 kDa (para spanning pg 876-877) and Kaiser-Alexnat et al teach that Cry34Ab1 and Cry35Ab1 bind to receptors in the Western corn rootworm gut with molecular weights of 110 kDa and 50kDa, respectively (Abstract). This demonstrates that Cry1F and Cry32Ab1/Cry35Ab1 bind to different receptors in the gut. If Cry1F binds a separate receptor than Cry32Ab1/Cry35Ab1, then a mutation in the receptor of an insect hindgut would not nullify the effects of all of the Cry toxins.

Claim 4 requires a plant comprising the sequence set forth in SEQ ID NO:6 and Claim 14 requires an isolated nucleic acid molecule comprising SEQ ID NO:6.

Considering that the combined references of Bing et al and Barbour et al teach an isolated DNA construct comprising the four expression cassettes as well as a plant comprising the construct, the construct comprising the polynucleotide of SEQ ID NO:6 is made obvious, as is a plant comprising the construct. Any variations that may occur in choosing the linking sequences between the regulatory or coding elements are merely design choices.

11. **Claims 28 and 31-32** are rejected under 35 U.S.C. 103(a) as being unpatentable over Bing et al (U.S. PG PUB 2008/0178357 A1) in view of Barbour et al (WO

Application/Control Number: 12/970,052

Page 10

Art Unit: 1638

2004099447 A2), further in view of Castle et al (2006. Curr Opin Biotechnol. 17:105-112), Hua et al (2001. Appl Environ Microbiol. 67(2):872-879), and Kaiser-Alexnat et al (2009. Insect Path Insect Parasit Nematodes. 45:235-238) as applied to claims 1-4 and 14 above, and further in view of Hall et al (U.S. Pat 6,222,106).

The claims are drawn to a method of producing hybrid corn seed wherein one of the two inbred corn lines that are planted comprise the DNA construct of Claim 1 (discussed above), wherein the plants of the first inbred corn line are the female parents or the male parents; the hybrid seed.

Bing et al and Barbour et al in view of Castle et al teach the DNA construct, as discussed above.

Bing et al in view of Barbour et al, further in view of Castle et al, Hua et al, and Kaiser-Alexnat et al do not teach a method of producing hybrid corn seed wherein one of the two inbred corn lines that are planted comprise the DNA construct of Claim 1 (discussed above), wherein the plants of the first inbred corn line are the female parents or the male parents and one of the two lines feature the emasculation of flowers; the hybrid seed.

Hall et al teach the active steps of producing hybrid corn seed, as well as the hybrid seed produced by the method (Claims 20 and 22). The remaining limitation is wherein the plants of the first inbred line are either the male or the female parents. Considering that there is no other option, Hall et al must teach at least one of the two options of male or female parents.

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 11

One would have been motivated to produce hybrid corn seed of two lines to increase the genetic variety in agricultural germplasm through intermingling the genotype of the line containing the event with any other corn line to increase fitness for agricultural production. The methods of producing hybrid corn seed are incredibly well known in the art.

***Allowable Subject Matter***

Claim 15 is allowed.

Claims 1-14 and 16-32 are rejected.

The following is a statement of reasons for the indication of allowable subject matter: The corn event DP-004114-3 is not known in the prior art. The event is described as a DNA construct comprising four operably linked cassettes comprising three Cry toxins and a phosphinothricin resistance gene, wherein the construct is flanked SEQ ID NO:27 and 28 (20mers) in a corn plant.

The closest prior art to the event sequences are Morris et al (PG PUB 2002/0182586 A1) which teach 87% sequence identity to SEQ ID NO:27 (alignment below) and Wang et al (PG PUB 2004/0181048 A1) which teach 87% sequence identity to SEQ ID NO:28 (alignment below).

US-10-087-192-997  
; Sequence 997, Application US/10087192  
; Publication No. US20020182586A1  
; GENERAL INFORMATION:  
; APPLICANT: Morris, David W.



Application/Control Number: 12/970,052

Page 12

Art Unit: 1638

```
; APPLICANT: Engelhard, Eric K.
; TITLE OF INVENTION: NOVEL COMPOSITIONS AND METHODS FOR
; TITLE OF INVENTION: CANCER
; FILE REFERENCE: 529452000122
; CURRENT APPLICATION NUMBER: US/10/087,192
; CURRENT FILING DATE: 2002-03-01
; PRIOR APPLICATION NUMBER: US 09/747,377
; PRIOR FILING DATE: 2000-12-22
; PRIOR APPLICATION NUMBER: US 09/798,586
; PRIOR FILING DATE: 2001-03-02
; NUMBER OF SEQ ID NOS: 2059
; SOFTWARE: FastSEQ for Windows Version 4.0
; SEQ ID NO 997
; LENGTH: 143412
; TYPE: DNA
; ORGANISM: Mus musculus
; FEATURE:
; NAME/KEY: misc_feature
; LOCATION: (1)...(143412)
; OTHER INFORMATION: n = A,T,C or G
US-10-087-192-997
```

```
Query Match          87.0%; Score 17.4; DB 7; Length 143412;
Best Local Similarity 94.7%;
Matches 18; Conservative 0; Mismatches 1; Indels 0; Gaps 0;
```

```
QY          2 AACACAGATCTGATAGTTT 20
              ||||| |||||
Db          39579 AACACAGCTCTGATAGTTT 39597
```

```
US-09-925-065A-292591
; Sequence 292591, Application US/09925065A
; Publication No. US20040181048A1
; GENERAL INFORMATION:
; APPLICANT: Wang, David G.
; TITLE OF INVENTION: Identification and Mapping of Single
; TITLE OF INVENTION: Nucleotide Polymorphisms in the Human Genome
; FILE REFERENCE: 108827.135
; CURRENT APPLICATION NUMBER: US/09/925,065A
; CURRENT FILING DATE: 2001-08-08
; PRIOR APPLICATION NUMBER: US 60/243,096
; PRIOR FILING DATE: 2000-10-24
; PRIOR APPLICATION NUMBER: US 60/252,147
; PRIOR FILING DATE: 2000-11-20
; PRIOR APPLICATION NUMBER: US 60/250,092
; PRIOR FILING DATE: 2000-11-30
; PRIOR APPLICATION NUMBER: US 60/261,766
; PRIOR FILING DATE: 2001-01-16
; PRIOR APPLICATION NUMBER: US 60/289,846
; PRIOR FILING DATE: 2001-05-09
; NUMBER OF SEQ ID NOS: 957086
; SOFTWARE: FastSEQ for Windows Version 4.0
; SEQ ID NO 292591
; LENGTH: 604
```

Application/Control Number: 12/970,052

Page 13

Art Unit: 1638

; TYPE: DNA  
; ORGANISM: Homo sapiens  
US-09-925-065A-292591

Query Match 87.0%; Score 17.4; DB 4; Length 604;  
Best Local Similarity 94.7%;  
Matches 18; Conservative 0; Mismatches 1; Indels 0; Gaps 0;

Qy 2 GTCAATTTGGAACAAGTGG 20  
|| |||||  
Db 306 GTGAATTTGGAACAAGTGG 324

### ***Conclusion***

Claims 1-14 and 16-32 are rejected.

Claims 5-13 and 15-27, and 29-30 are free of the prior art.

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jeff Bolland, Ph.D. whose telephone number is (571) 272-6750. The examiner can normally be reached on Monday - Friday 8:00am - 5:00pm Eastern Standard Time.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisors, Anne Marie Grunberg and Joe Zhou can be reached on (571) 272-0975 and (571) 272-0724, respectively. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 14

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/Jeffrey Bolland/  
Examiner, Art Unit 1638

/Anne R. Kubelik/  
Primary Examiner, Art Unit 1638

*nr*

# **Exhibit I**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application No. : 12/970,052 Confirmation No. : 5362  
Applicant : Scott Diehn  
Filed : December 16, 2010  
TC/A.U. : 1638  
Examiner : Jeffery R. Bolland  
Docket No. : 3700  
Customer No. : 27310  
Title : Maize Event DP-004114-3 and Methods of Detecting Thereof

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

AMENDMENT UNDER 37 C.F.R. § 1.111

In response to the Office Action of February 04, 2013, please amend the above-identified application as follows:

**Amendments to the Specification** begin on page 2 of this paper.

**A Complete Listing of the Claims** begins on page 3 of this paper.

**Remarks/Arguments** begin on page 11 of this paper.

Appl. No.: 12/970,052  
Amdt. Dated 05/03/2013  
Reply to Office Action of February 04, 2013

**AMENDMENTS TO THE SPECIFICATION:**

Please replace the paragraphs from page 52, line 7 to page 53, line 20 with the following amended paragraphs:

To characterize the inserted T-DNA in 4114 maize, PCR primers were designed to amplify the T-DNA insert in six separate, overlapping PCR products as outlined in Table 7: fragments A through F (Positions indicated in Figure 5). As expected, the predicted PCR products were generated only from 4114 maize genomic DNA samples, and were not present in the control maize samples. The six PCR products were cloned and sequenced. When comparing the sequence of the inserted T-DNA in 4114 maize to the T-DNA region of plasmid PHP27118 used to create 4114 maize, it was determined that there was a 29 bp deletion on the RB end, and a 24 bp deletion on the LB end. RB and LB termini deletions often occur in *Agrobacterium*-mediated transformation (Kim *et al.* (2007) *Plant J.* 51:779-791). All remaining sequence is intact and identical to that of plasmid PHP27118. The sequence of the insertion is presented in ~~SEQ ID NO: 27~~ **SEQ ID NO: 6**.

To verify the additional 5' genomic border sequence, PCR was performed with a forward primer (SEQ ID NO: 11) in the 5' genomic border region and a reverse primer (SEQ ID NO: 12) within the inserted T-DNA. The resulting 2,511 bp PCR fragment A from 4114 maize genomic DNA samples was cloned and sequenced (Figure 3). The 2,422 bp of the 5' genomic border region sequence is set forth in nucleotides 1-2,422 of ~~SEQ ID NO: 27~~ **SEQ ID NO: 6**.

To verify the additional 3' genomic border sequence, PCR was performed with a forward primer (SEQ ID NO: 21) within the inserted T-DNA and a reverse primer (SEQ ID NO: 22) in the 3' genomic border region. The resulting 2,612 bp PCR fragment F from 4114 maize genomic DNA samples was cloned and sequenced (Figure 3). The 2,405 bp of the 3' genomic border region sequence is set forth in nucleotides 14,348 to 16,752 of ~~SEQ ID NO: 27~~ **SEQ ID NO: 6**.



Appl. No.: 12/970,052  
Amdt. Dated 05/03/2013  
Reply to Office Action of February 04, 2013

Amendments to the Claims:

1. (currently amended) A DNA construct comprising: a first, second, third and fourth expression cassette, wherein said first expression cassette in operable linkage comprises:

- (a) a maize ubiquitin promoter;
- (b) a 5' untranslated exon of a maize ubiquitin gene;
- (c) a maize ubiquitin first intron;
- (d) a Cry1F encoding DNA molecule; and
- (e) a poly(A) addition signal from ORF 25 terminator;

said second expression cassette in operable linkage comprises:

- (1) a maize ubiquitin promoter;
- (2) a 5' untranslated exon of a maize ubiquitin gene;
- (3) a maize ubiquitin first intron;
- (4) a Cry34Ab1 encoding DNA molecule; and
- (5) a PinII transcriptional terminator;

said third expression cassette **in operable linkage comprises;** ~~comprising in operable linkage~~

- (i) a wheat peroxidase promoter;
- (ii) a Cry35Ab1 encoding DNA molecule; and
- (iii) a PinII transcriptional terminator; and

said fourth expression cassette **in operable linkage comprises;** ~~comprising in operable linkage~~

- (a) a CaMV 35S promoter;
- (b) a *pat* encoding DNA molecule; and
- (c) a 3' transcriptional terminator from CaMV 35S;

**wherein the DNA construct is flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28.**

Appl. No.: 12/970,052

Amdt. Dated 05/03/2013

Reply to Office Action of February 04, 2013

2. (currently amended) A plant comprising the DNA construct of claim 1, **wherein the DNA construct is flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28.**

3. (original) A plant of claim 2, wherein said plant is a corn plant.

4. (original) A plant comprising the sequence set forth in SEQ ID NO: 6.

5. (currently amended) A corn plant comprising the genotype of the corn event DP-004114-3 **deposited with American Type Culture Collection (ATCC) under Accession No. PTA-11506**, wherein said genotype comprises the **DNA construct of claim 1 flanked by the** nucleotide sequence set forth in SEQ ID NO: 27 and SEQ ID NO: 28.

Claims 6. and 7. (cancelled)

8. (original) A corn event DP-004114-3, wherein a representative sample of seed of said corn event has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

9. (original) Plant parts of the corn event of claim 8.

10. (currently amended) Seed comprising corn event DP-004114-3, wherein said seed comprises **the DNA construct of claim 1 flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28** ~~a DNA molecule selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28~~, wherein a representative sample of corn event DP-004114-3 seed of has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

11. (original) A corn plant, or part thereof, grown from the seed of claim 10.

12. (original) A transgenic seed produced from the corn plant of claim 11 comprising event DP-004114-3.

13. (original) A transgenic corn plant, or part thereof, grown from the seed of claim 12.

14. (original) An isolated nucleic acid molecule comprising a nucleotide sequence selected from the group consisting of SEQ ID NO: 6; SEQ ID NO: 27; SEQ ID NO: 28, and full length complements thereof.

15. (original) An amplicon comprising the nucleic acid sequence selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28 and full length complements thereof.

Appl. No.: 12/970,052

Amdt. Dated 05/03/2013

Reply to Office Action of February 04, 2013

16. (currently amended) A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence ~~which is or is complementary to a sequence~~ selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28 **or the complement thereof**, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed of has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

17. (original) The biological sample of claim 16, wherein said biological sample comprise plant, tissue, or seed of transgenic corn event DP-004114-3.

18. (original) The biological sample of claim 17, wherein said biological sample is a DNA sample extracted from the transgenic corn plant event DP-004114-3, and wherein said DNA sample comprises one or more of the nucleotide sequences selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28, and the complement thereof.

19. (original) The biological sample of claim 18, wherein said biological sample is selected from the group consisting of corn flour, corn meal, corn syrup, corn oil, corn starch, and cereals manufactured in whole or in part to contain corn by-products.

20. (currently amended) An extract derived from corn event DP-004114-3 plant, tissue, or seed and comprising a nucleotide sequence ~~which is or is complementary to a sequence~~ selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28 **or the complement thereof**, wherein a representative sample of said corn event DP-004114-3 seed has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

21. (original) The extract of claim 20, wherein said nucleotide sequence is detectable in said extract using a nucleic acid amplification or nucleic acid hybridization method.

22. (original) The extract of claim 21, wherein said extract comprises plant, tissue, or seed of transgenic corn plant event DP-004114-3.

23. (original) The extract of claim 22, further comprising a composition selected from the group consisting of corn flour, corn meal, corn syrup, corn oil, corn starch, and cereals manufactured in whole or in part to contain corn by-products, wherein said composition comprises a detectable amount of said nucleotide sequence.

24. (currently amended) A method of producing hybrid corn seeds comprising:

Appl. No.: 12/970,052

Amdt. Dated 05/03/2013

Reply to Office Action of February 04, 2013

- (a) planting seeds of a first inbred corn line comprising **the DNA construct of claim 1 flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28** ~~a nucleotide sequence selected from the group consisting of SEQ ID NO: 27, SEQ ID NO: 28~~ and seeds of a second inbred line having a different genotype;
- (b) cultivating corn plants resulting from said planting until time of flowering;
- (c) emasculating said flowers of plants of one of the corn inbred lines;
- (d) sexually crossing the two different inbred lines with each other; and
- (e) harvesting the hybrid seed produced thereby.

25. (original) The method according to claim 24, wherein the plants of the first inbred corn line are the female parents.

26. (original) The method according to claim 24, wherein the plants of first inbred corn line are the male parents.

27. (currently amended) A method for producing a corn plant resistant to lepidopteran pests comprising:

- (a) sexually crossing a first parent corn plant with a second parent corn plant, wherein said first or second parent corn plant comprises event DP-004114-3 DNA, **deposited under Accession No. PTA-11506 with American Type Culture Collection (ATCC)**, thereby producing a plurality of first generation progeny plants;
- (b) selecting a first generation progeny plant that is resistant to lepidopteran insect infestation;
- (c) selfing the first generation progeny plant, thereby producing a plurality of second generation progeny plants; and
- (d) selecting from the second generation progeny plants, a plant that is resistant to lepidopteran pests;

wherein the second generation progeny plants comprise the DNA construct according to claim 1.

28. (currently amended) A method of producing hybrid corn seeds comprising:

Appl. No.: 12/970,052

Amdt. Dated 05/03/2013

Reply to Office Action of February 04, 2013

- (a) planting seeds of a first inbred corn line comprising the DNA construct of claim 1 **flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28** and seeds of a second inbred line having a genotype different from the first inbred corn line;
- (b) cultivating corn plants resulting from said planting until time of flowering;
- (c) emasculating said flowers of plants of one of the corn inbred lines;
- (d) sexually crossing the two different inbred lines with each other; and
- (e) harvesting the hybrid seed produced thereby.

29. (currently amended) The method of claim 28 further comprising ~~the step of~~ backcrossing the second generation progeny plant of step (d) that comprises corn event DP-004114-3 DNA, **deposited under Accession No. PTA-11506 with American Type Culture Collection (ATCC)**, to the parent plant that lacks the corn event DP-004114-3 DNA, thereby producing a backcross progeny plant that is resistant to at least western corn rootworm.

30. (currently amended) A method for producing a corn plant resistant to at least corn rootworm, said method comprising:

- (a) sexually crossing a first parent corn plant with a second parent corn plant, wherein said first or second parent corn plant is a corn event DP-004114-3, **deposited under Accession No. PTA-11506 with American Type Culture Collection (ATCC)**, plant, thereby producing a plurality of first generation progeny plants;
- (b) selecting a first generation progeny plant that is resistant to at least corn rootworm infestation;
- (c) backcrossing the first generation progeny plant of step (b) with the parent plant that lacks corn event DP-004114-3 DNA, thereby producing a plurality of backcross progeny plants; and
- (d) selecting from the backcross progeny plants, a plant that is resistant to at least corn rootworm infestation;

wherein the selected backcross progeny plant of step (d) comprises SEQ ID NO: 6.

31. (original) The method according to claim 28, wherein the plants of the first inbred corn line are the female parents or male parents.

Appl. No.: 12/970,052

Amdt. Dated 05/03/2013

Reply to Office Action of February 04, 2013

32. (original) Hybrid seed produced by the method of claim 28.

33. (withdrawn) A method of determining zygosity of DNA of a corn plant comprising corn event DP-004114-3 in a biological sample comprising:

(a) contacting said sample with a first primer selected from the group consisting of SEQ ID NOs: 11, 13, 23 or 24, and a second primer selected from the group consisting of SEQ ID NOs: 20, 22, 25 or 26, such that

(1) when used in a nucleic acid amplification reaction comprising corn event DP-004114-3 DNA, produces a first amplicon that is diagnostic for corn event DP-004114-3, and

(2) when used in a nucleic acid amplification reaction comprising corn genomic DNA other than DP-004114-3 DNA, produces a second amplicon that is diagnostic for corn genomic DNA other than DP-004114-3 DNA;

(b) performing a nucleic acid amplification reaction; and

(c) detecting the amplicons so produced, wherein detection of presence of both amplicons indicates that said sample is heterozygous for corn event DP-004114-3 DNA, wherein detection of only the first amplicon indicates that said sample is homozygous for corn event DP-004114-3 DNA.

34. (withdrawn) A method of detecting the presence of a nucleic acid molecule that is unique to event DP-004114-3 in a sample comprising corn nucleic acids, the method comprising:

(a) contacting the sample with a pair of primers that, when used in a nucleic-acid amplification reaction with genomic DNA from event DP-004114-3 produces an amplicon that is diagnostic for event DP-004114-3;

(b) performing a nucleic acid amplification reaction, thereby producing the amplicon; and

(c) detecting the amplicon.

35. (withdrawn) A pair of polynucleotide primers comprising a first polynucleotide primer and a second polynucleotide primer which function together in the presence of an event DP-004114-3 DNA template in a sample to produce an amplicon diagnostic for event DP-004114-3.



Appl. No.: 12/970,052

Amdt. Dated 05/03/2013

Reply to Office Action of February 04, 2013

36. (withdrawn) The pair of polynucleotide primers according to claim 35, wherein the sequence of the first polynucleotide primer is or is complementary to a corn plant genome sequence flanking the point of insertion of a heterologous DNA sequence inserted into the corn plant genome of event DP-004114-3, and the sequence of the second polynucleotide primer is or is complementary to the heterologous DNA sequence inserted into the genome of event DP-004114-3.

37. (withdrawn) The pair of polynucleotide primers according to claim 36, wherein

- (a) the first polynucleotide primer comprises at least 10 contiguous nucleotides of a nucleotide sequence selected from the group consisting of nucleotides 1-2422 of SEQ ID NO: 6, nucleotides 14348-16752 of SEQ ID NO: 6, and the complements thereof; and
- (b) the second polynucleotide primer comprises at least 10 contiguous nucleotides from nucleotides 2423-14347 of SEQ ID NO: 6, or the complements thereof.

38. (withdrawn) The pair of polynucleotide primers according to claim 37, wherein

- (a) the first polynucleotide primer comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 20, SEQ ID NOs: 22-26 and the complements thereof; and
- (b) the second polynucleotide primer comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 12, SEQ ID NOs: 14-19, SEQ ID NO: 21, and the complements thereof.

39. (withdrawn) The primer pair of claim 37, wherein said first primer and said second primer are at least 18 nucleotides.

40. (withdrawn) The primer pair of claim 37, wherein said first primer and said second primer are at least 24 nucleotides.

41. (withdrawn) A method of detecting the presence of DNA corresponding to the DP-004114-3 event in a sample, the method comprising:

- (a) contacting the sample comprising maize DNA with a polynucleotide probe that hybridizes under stringent hybridization conditions with DNA from maize event DP-004114-3 and does not hybridize under said stringent hybridization conditions with a non- DP-004114-3 maize plant DNA;
- (b) subjecting the sample and probe to stringent hybridization conditions; and

Appl. No.: 12/970,052

Amdt. Dated 05/03/2013

Reply to Office Action of February 04, 2013

(c) detecting hybridization of the probe to the DNA;

wherein detection of hybridization indicates the presence of the DP-004114-3 event.

42. (withdrawn) A kit for detecting nucleic acids that are unique to event DP-004114-3 comprising at least one nucleic acid molecule of sufficient length of contiguous polynucleotides to function as a primer or probe in a nucleic acid detection method, and which upon amplification of or hybridization to a target nucleic acid sequence in a sample followed by detection of the amplicon or hybridization to the target sequence, are diagnostic for the presence of nucleic acid sequences unique to event DP-004114-3 in the sample.

43. (withdrawn) The kit according to claim 42, wherein the nucleic acid molecule comprises a nucleotide sequence from SEQ ID NO: 6.

44. (withdrawn) The kit according to claim 43, wherein the nucleic acid molecule is a primer selected from the group consisting of SEQ ID NOs: 11-26, and the complements thereof.

Appl. No.: 12/970,052  
Amdt. Dated 05/03/2013  
Reply to Office Action of February 04, 2013

## **REMARKS**

### ***Status of the Claims***

Claims 1-5 and 8-44 are pending are pending. Claims 6 and 7 are presently cancelled. Claims 33-44 have been withdrawn from consideration.

Claim 1 has been amended to insert a “semicolon” at the end of the lines beginning “said third expression” and “said forth expression” as suggested by the Examiner. For further clarity Applicant has also amended these lines beginning “said third expression” and “said forth expression” of Claim 1 to recite the “expression cassette in operable linkage comprises” to make said lines consistent with the preceding similar lines. These amendments to claim 1 are formal in nature and thus, do not introduce new matter.

Claims 1, 2, 5, 10, 24, 28 have been amended to recite that the “... DNA construct is flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28.” Support for this amendment can found throughout the specification, at least on p. 13, lines 6-9; SEQ ID NO: 6; and Figure 5.

Claims 16 and 20 have been amended to clarify that the sequence of SEQ ID NO: 27 and SEQ ID NO: 28 are distinct from the complement as suggested by the Examiner. This amendment to claims 16 and 20 is formal in nature and thus, does not introduce new matter.

Claims 5, 27, 29, and 30 have been amended to reference the DP-004114-3 event by the ATCC deposit accession number as required by the Examiner. This amendment to claim 5, 27, 29, and 30 is formal in nature and thus, does not introduce new matter.

### ***Amendments to the Specification***

Applicant has amended the specification to correct the typographical error in the SEQ ID NO: identifier occurring on page 3, line 8, line 14, and line 20. Applicant has amended said to SEQ ID NO: 6. From the specification (p. 13 lines 6-9) and the sequence listing SEQ ID NO: 27 is the junction sequence point between the maize genomic DNA and the 5' end of the insert and is 20 nucleotides in length. SEQ ID NO: 6 is the total (16,752 bp) genomic 5', 3' genomic border sequences flanking the insert that was sequenced (p. 47, lines 18-28). The amended paragraphs as filed refer to this 16,752 bp sequence, which is SEQ ID NO: 6 and not SEQ ID NO: 27. Applicant request that amended paragraphs be substituted into specification. No new matter has been added.

Appl. No.: 12/970,052  
Amdt. Dated 05/03/2013  
Reply to Office Action of February 04, 2013

### ***Claim Objections***

As suggested by the Examiner the lines beginning “said third expression cassette” “said fourth expression cassette” of claim 1 have been amended to clarify the claim to recite “ ... expression cassette in operable linkage comprises: ... ”.

### ***Claim Rejections – 35 USC § 112 second paragraph***

**Claims 5-7, 16-23, 27, and 29-30** are rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor. Claims 5, 16, 20, 27, 29, and 30 have been amended. Claims 6 and 7 have been cancelled. This rejection is respectfully traversed.

The Action alleges **Claim 5** is indefinite as the event name is an arbitrary designation in the absence of a deposit organization and accession number such as American Type Culture Collection (ATCC) with Accession No. PTA-004114-3.

Applicant has amended claim 5 to refer to the ATCC deposit as suggested by the Examiner and to recite the “genotype comprises the DNA construct of claim 1 flanked by the nucleotide sequence set forth in SEQ ID NO: 27 and SEQ ID NO: 28. Claims 6 and 7 are presently cancelled.

The Action alleges **Claims 16 and 20** are indefinite in the recitation of "...a nucleotide sequence which is or is complementary to a sequence selected from the group consisting of...".

Applicant has amended claims 16 and 20 to recite “ ... nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28 or the complement thereof ...”.

The Action alleges **Claims 27 and 29-30** are indefinite in the recitation of "...event DP-004114-3...".

Applicant has amended claims 27, 29 and 30 to refer to the ATCC deposit as required by the Examiner.

The Action alleges **Claim 29** there is insufficient antecedent basis for the limitation "the step" in line 1 in the claim.

Appl. No.: 12/970,052  
Amdt. Dated 05/03/2013  
Reply to Office Action of February 04, 2013

To overcome the rejection Applicant has amended claim 29 to delete the phrase “the step of”.

In view of the amendment to the claims and the above remarks, it is submitted that the rejections of the claims under 35 USC § 112 second paragraph, are obviated and should be withdrawn.

***Claim Rejections – 35 USC § 112 first paragraph***

**Claims 5-13 and 16-32** are rejected under 35 U.S.C. 112, first paragraph, as allegedly failing to comply with the enablement requirement. The claim(s) allegedly contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention. Claim 24 has been amended. This rejection is respectfully traversed.

The Action states the specification discloses the deposit of corn seed with the American Type Culture Collection, ATCC Deposit Number PTA- 11506, however no statement has been provided stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent.

Applicant respectfully disagrees with this position of the Examiner and submits that requirements of 37 CFR § 1.801 -1.809 are fully met by the information that is provided in the paragraph from line 2 of page 10 to line 5 of page 11. Specifically, the specification states: *“Upon allowance of any claims in the application, the Applicant(s) will make available to the public, pursuant to 37 C.F.R. § 1.808, sample(s) of the deposit of at least 2500 seeds of hybrid maize ...”* (p. 10, lines 15-17). 37 CFR § 1.808 (a)(2) states: *“all restrictions imposed by the depositor on the availability to the public of the deposited material will be irrevocably removed upon the granting of the patent”*.

Accordingly, the Applicant respectfully submits that said statement is fully compliant of the requirements of 37 CFR § 1.801-1.809 and Applicant requests that the rejection under 35 USC § 112 first paragraph be withdrawn.

The Action asserts **Claims 24-26** drawn to a method of producing hybrid corn seed comprising sequences of SEQ ID NO: 27 and 28 do not encode any of the Cry proteins or the

Appl. No.: 12/970,052  
Amdt. Dated 05/03/2013  
Reply to Office Action of February 04, 2013

phosphinothricin resistance gene and asserts the Applicant has failed to provide any guidance on a plant that comprises SEQ ID NO: 27 and 28 but not the DNA construct comprising the four expression cassettes.

Applicant has amended claim 24 to recite a “... first inbred corn line comprising a DNA construct of claim 1, wherein the DNA construct is flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28”.

The Action also states the specification allegedly fails to provide enablement for the genotype of the corn even DP-004114-3 wherein the genotype comprises the four expression cassettes flanked by SEQ ID NO: 27 and 28.

Applicant respectfully disagrees with this position of the Examiner on the enablement of the specification for the genotype of the corn even DP-004114-3 wherein the genotype comprises the four expression cassettes flanked by SEQ ID NO:27 and 28. Example 4 discloses the sequence characterization of the insert and genomic border regions of event DP-004114-3. In total, 16,752 bp of 4114 maize genomic sequence (SEQ ID NO: 6) was confirmed, comprising 2,422 bp of the 5' genomic border sequence, 2,405 bp of the 3' genomic border sequence, and 11,925 bp of inserted T-DNA from PHP27118. The 5' and 3' genomic border regions of 4114 maize were verified to be of maize origin by PCR amplification and sequencing of the genomic border regions from both 4114 maize and control maize plants. All 4114 maize plants were positive for the event-specific PCR and the PAT, Cry1F, and Cry34Ab1 proteins, whereas all the control maize plants were negative (Table 6). The T-DNA sequence information of plasmid PHP27118 was used to design primers to verify the inserted sequence in 4114 maize (Tables 7 and 8).

Therefore, Applicant submits that the specification is enabling for the genotype of corn comprising the DP-004114-3 event and in light of the amendments and the teaching of the specification.

In view of the amendments and above remarks, it is respectfully submitted that rejection of claims claims 5-13 and 16-32 under 35 USC § 112 first paragraph should be withdrawn.

### ***Claim Rejections - 35 USC § 103***

**Claims 1-4 and 14** are rejected under 35 U.S.C. 103(a) as allegedly being unpatentable over Bing et al (U.S. PG PUB 2008/0178357 A1) in view of Barbour et al (WO2004099447 A2), further in view of each of Castle et al (2006. Curr Opin Biotechno. 17:105-112), Hua et al (2001.



Appl. No.: 12/970,052  
Amdt. Dated 05/03/2013  
Reply to Office Action of February 04, 2013

Appl Environ Microbiol. 67(2):872-879), and Kaiser-Alexnat et al (2009. Insect Path Insect Parasit Nematodes. 45:235-238). Claims 1 and 2 have been amended. This rejection is respectfully traversed.

Without acquiescing as to the merits of the rejection and solely to facilitate prosecution Applicant has amended claims 1 and 2 to recite that the DNA construct is flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28.

Claim 14 is directed to an isolated nucleic acid molecule of SEQ ID NO: 6; SEQ ID NO: 27 or SEQ ID NO: 28 and Claim 4 is directed to a plant comprising the sequence of SEQ ID NO: 6 that comprises the DNA construct comprising the four operably linked cassettes comprising three Cry toxins and a phosphinothricin resistance gene and 2,422 bp of the 5' genomic border region sequence set forth in nucleotides 1-2,422 of SEQ ID NO: 6 and the 2,405 bp of the 3' genomic border region sequence is set forth in nucleotides 14,348 to 16,752 of SEQ ID NO: 6, which respectively are the junction points into the genome as represented by SEQ ID NO: 27 and SEQ ID NO: 28. Therefore, Bing et al in view of Barbour et al, further in view of each of Castle et al and Kaiser-Alexnat et al do not teach all of the claim elements since they don't teach the insertion point of the instant DP-004114-3 event.

The Action states that Claim 15 is allowable and acknowledges the corn event DP-004114-3 is not known in the prior art where the event is described as a DNA construct comprising four operably linked cassettes comprising three Cry toxins and a phosphinothricin resistance gene, wherein the construct is flanked by SEQ ID NO: 27 and 28. The Action also states that Claims 5-13 and 15-27, and 29-30 are free of the prior art.

In view of the amendments to the claims, the arguments set forth above, and the Actions acknowledgement that the corn event DP-004114-3 is not known in the prior art, Applicant respectfully submits that the presently claimed subject matter is non-obvious over Bing et al in view of Barbour et al, further in view of each of Castle et al and Kaiser-Alexnat et al. and the rejection under 35 USC § 103 be withdrawn.

Appl. No.: 12/970,052  
Amdt. Dated 05/03/2013  
Reply to Office Action of February 04, 2013

### **CONCLUSIONS**

In view of the foregoing remarks and amendments, the Examiner is respectfully requested to withdraw the rejections made to the pending claims. Applicants respectfully submit that this application is now ready for allowance. Early notice to this effect is solicited.

Should the Examiner have further questions or comments with respect to examination of this case, it is respectfully requested that the Examiner telephone the undersigned so that further examination of this application can be expedited.

It is not believed that extensions of time or fees for net addition of claims are required, beyond those that may otherwise be provided for in documents accompanying this paper. However, in the event that additional extensions of time are necessary to allow consideration of this paper, such extensions are hereby petitioned under 37 CFR § 1.136(a), and any fee required therefore (including fees for net addition of claims) is hereby authorized to be charged to Deposit Account No. 16-1852.

Respectfully submitted,

/s. christopher bauer/

S. Christopher Bauer  
Agent for Applicant(s)  
Registration No. 42,305

PIONEER HI-BRED INTERNATIONAL, INC.  
Intellectual Property Group  
7250 N.W. 62<sup>nd</sup> Avenue  
P.O. Box 552  
Johnston, Iowa 50131-0552  
Phone: (515) 535-0060  
Facsimile: (515) 535-6883

# **Exhibit J**



## UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE  
 United States Patent and Trademark Office  
 Address: COMMISSIONER FOR PATENTS  
 P.O. Box 1450  
 Alexandria, Virginia 22313-1450  
 www.uspto.gov

## NOTICE OF ALLOWANCE AND FEE(S) DUE

27310 7590 07/01/2013  
 PIONEER HI-BRED INTERNATIONAL, INC.  
 7250 N.W. 62ND AVENUE  
 P.O. BOX 552  
 JOHNSTON, IA 50131-0552

EXAMINER

BOLLAND, JEFFREY R

ART UNIT

PAPER NUMBER

1638

DATE MAILED: 07/01/2013

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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12/970,052

12/16/2010

Scott Diehn

3700

5362

TITLE OF INVENTION: MAIZE EVENT DP-004114-3 AND METHODS FOR DETECTION THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1780	\$300	\$0	\$2080	10/01/2013

**THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.**

**THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.**

**HOW TO REPLY TO THIS NOTICE:**

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

**IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.**

PART B - FEE(S) TRANSMITTAL

# 7495

Complete and send this form, together with applicable fee(s), to: **Mail**

**Mail Stop ISSUE FEE**  
**Commissioner for Patents**  
**P.O. Box 1450**  
**Alexandria, Virginia 22313-1450**  
**or Fax (571)-273-2885**

INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

CURRENT CORRESPONDENCE ADDRESS (Note: Use Block 1 for any change of address)

Note: A certificate of mailing can only be used for domestic mailings of the Fee(s) Transmittal. This certificate cannot be used for any other accompanying papers. Each additional paper, such as an assignment or formal drawing, must have its own certificate of mailing or transmission.

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**Certificate of Mailing or Transmission**

I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/970,052	12/16/2010	Scott Diehn	3700	5362

TITLE OF INVENTION: MAIZE EVENT DP-004114-3 AND METHODS FOR DETECTION THEREOF

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1780	\$300	\$0	\$2080	10/01/2013

EXAMINER	ART UNIT	CLASS-SUBCLASS
BOLLAND, JEFFREY R	1638	800-302000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

1 \_\_\_\_\_

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

2 \_\_\_\_\_

3 \_\_\_\_\_

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

- ☐ Issue Fee  
☐ Publication Fee (No small entity discount permitted)  
☐ Advance Order - # of Copies \_\_\_\_\_

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)

- ☐ A check is enclosed.  
☐ Payment by credit card. Form PTO-2038 is attached.  
☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number \_\_\_\_\_ (enclose an extra copy of this form).

**5. Change in Entity Status** (from status indicated above)☐ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see form PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

☐ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

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NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

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Authorized Signature \_\_\_\_\_

Date \_\_\_\_\_

Typed or printed name \_\_\_\_\_

Registration No. \_\_\_\_\_

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This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/970,052	12/16/2010	Scott Diehn	3700	5362

27310 7590 07/01/2013  
 PIONEER HI-BRED INTERNATIONAL, INC.  
 7250 N.W. 62ND AVENUE  
 P.O. BOX 552  
 JOHNSTON, IA 50131-0552

EXAMINER

BOLLAND, JEFFREY R

ART UNIT PAPER NUMBER

1638

DATE MAILED: 07/01/2013

### Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)

(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 210 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 210 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

## Privacy Act Statement

**The Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

<b>Notice of Allowability</b>	<b>Application No.</b> 12/970,052	<b>Applicant(s)</b> DIEHN ET AL.	
	<b>Examiner</b> JEFFREY BOLLAND	<b>Art Unit</b> 1638	<b>AIA (First Inventor to File) Status</b> No

**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--**

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to Arguments and amendments filed 2 May 2013.  
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on \_\_\_\_.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on \_\_\_\_; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are 1-5, 8-21, 23-27, and 29-32. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see [http://www.uspto.gov/patents/init\\_events/pph/index.jsp](http://www.uspto.gov/patents/init_events/pph/index.jsp) or send an inquiry to [PPHfeedback@uspto.gov](mailto:PPHfeedback@uspto.gov).
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a) ☐ All    b) ☐ Some    \*c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_.
3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

\* Certified copies not received: \_\_\_\_.

**Interim copies:**

a) ☐ All    b) ☐ Some    c) ☐ None of the: Interim copies of the priority documents have been received.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.  
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS ( as "replacement sheets") must be submitted.  
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date \_\_\_\_.

**Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

<ol style="list-style-type: none"> <li>1. <input type="checkbox"/> Notice of References Cited (PTO-892)</li> <li>2. <input type="checkbox"/> Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date ____</li> <li>3. <input type="checkbox"/> Examiner's Comment Regarding Requirement for Deposit of Biological Material</li> <li>4. <input checked="" type="checkbox"/> Interview Summary (PTO-413), Paper No./Mail Date <u>20130605</u>.</li> </ol>	<ol style="list-style-type: none"> <li>5. <input checked="" type="checkbox"/> Examiner's Amendment/Comment</li> <li>6. <input type="checkbox"/> Examiner's Statement of Reasons for Allowance</li> <li>7. <input type="checkbox"/> Other ____.</li> </ol>
--	---

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 2

### **EXAMINER'S AMENDMENT**

An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Christopher Bauer agreed to the Examiner's amendment in a telephone interview on 10 June 2013.

IN THE CLAIMS:

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 3

Claims 22, 28, and 33-44 are cancelled without prejudice.

1. A DNA construct comprising: a first, second, third and fourth expression cassette, wherein said first expression cassette in operable linkage comprises:

- (a) a maize ubiquitin promoter;
- (b) a 5' untranslated exon of a maize ubiquitin gene;
- (c) a maize ubiquitin first intron;
- (d) a Cry1F encoding DNA molecule; and
- (e) a poly(A) addition signal from ORF 25 terminator;

said second expression cassette in operable linkage comprises:

- (1) a maize ubiquitin promoter;
- (2) a 5' untranslated exon of a maize ubiquitin gene;
- (3) a maize ubiquitin first intron;
- (4) a Cry34Ab1 encoding DNA molecule; and
- (5) a PinII transcriptional terminator;

said third expression cassette in operable linkage comprises;

- (i) a wheat peroxidase promoter;
- (ii) a Cry35Ab1 encoding DNA molecule; and
- (iii) a PinII transcriptional terminator; and

said fourth expression cassette in operable linkage comprises;

- (a) a CaMV 35S promoter;

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 4

(b) a pat encoding DNA molecule; and

(c) a 3' transcriptional terminator from CaMV 35S;

wherein the ~~DNA construct is~~ four cassettes are flanked by the ~~5' junction sequence of~~  
SEQ ID NO: 27 at the 5' end and the ~~3' junction sequence of~~ SEQ ID NO: 28 at the 3'  
end.

2. A plant comprising the DNA construct of claim 1, ~~wherein the DNA construct is~~  
~~flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of~~  
~~SEQ ID NO: 28.~~

5. A corn plant comprising the genotype of the corn event DP-004114-3 deposited  
with American Type Culture Collection (ATCC) under Accession No. PTA-11506,  
wherein said genotype comprises the DNA construct of claim 1, ~~flanked by the~~  
~~nucleotide sequence set forth in SEQ ID NO: 27 and SEQ ID NO: 28.~~

8. A corn plant comprising the genotype of the A-corn event DP-004114-3, wherein  
a representative sample of seed of said corn event has been deposited with American  
Type Culture Collection (ATCC) with Accession No. PTA-11506.

9. A plant ~~Plant~~ parts of the plant comprising the corn event of claim 8.

10. A seed ~~Seed~~ comprising corn event DP-004114-3, wherein said seed comprises  
the DNA construct of claim 1 ~~flanked by the 5' junction sequence of SEQ ID NO: 27 and~~  
~~the 3' junction sequence of SEQ ID NO: 28~~, wherein a representative sample of corn  
event DP-004114-3 seed ~~of~~ has been deposited with American Type Culture Collection  
(ATCC) with Accession No. PTA-11506.

Application/Control Number: 12/970,052

Page 5

Art Unit: 1638

12. A transgenic seed produced from the corn plant of claim 11 ~~comprising event DP-004114-3~~, wherein the seed comprises corn event DP-004114-3.

16. A biological sample derived from corn event DP-004114-3 plant, tissue, or seed, wherein said sample comprises a nucleotide sequence selected from the group consisting of SEQ ID NO: 27 and SEQ ID NO: 28, or the complement thereof, wherein said nucleotide sequence is detectable in said sample using a nucleic acid amplification or nucleic acid hybridization method, wherein a representative sample of said corn event DP-004114-3 seed ~~of~~ has been deposited with American Type Culture Collection (ATCC) with Accession No. PTA-11506.

19. The biological sample of claim 18, wherein said biological sample is selected from the group consisting of corn flour, corn meal, ~~corn syrup, corn oil, corn starch~~, and cereals manufactured in whole or in part to contain corn by-products.

23. The extract of claim ~~22~~21, further comprising a composition selected from the group consisting of corn flour, corn meal, corn syrup, corn oil, corn starch, and cereals manufactured in whole or in part to contain corn by-products, wherein said composition comprises a detectable amount of said nucleotide sequence.

24. A method of producing hybrid corn seeds comprising:

- (a) planting seeds of a first inbred corn line comprising the DNA construct of claim 1 ~~flanked by the 5' junction sequence of SEQ ID NO: 27 and the 3' junction sequence of SEQ ID NO: 28~~ and seeds of a second inbred line having a ~~different~~ genotype different from the first inbred corn line;
- (b) cultivating corn plants resulting from said planting until time of flowering;



Application/Control Number: 12/970,052  
Art Unit: 1638

Page 6

- (c) emasculating said flowers of plants of one of the corn inbred lines;
- (d) sexually crossing the two different inbred lines with each other; and
- (e) harvesting the hybrid seed produced thereby.

29. The method of claim ~~28~~24 further comprising backcrossing the second generation progeny plant of step (d) that comprises corn event DP-004114-3 DNA, deposited under Accession No. PTA-11506 with American Type Culture Collection (ATCC), to the parent plant that lacks the corn event DP-004114-3 DNA, thereby producing a backcross progeny plant that is resistant to at least western corn rootworm.

31. The method according to claim ~~28~~24, wherein the plants of the first inbred corn line are the female parents or male parents.

32. Hybrid seed produced by the method of claim ~~28~~24.

### ***Conclusion***

Any inquiry concerning this communication or earlier communications from the examiner should be directed to Jeff Bolland, Ph.D. whose telephone number is (571) 272-6750. The examiner can normally be reached on Monday - Friday 8:00am - 5:00pm Eastern Standard Time.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisors, Anne Marie Grunberg and Joe Zhou can be reached on (571) 272-0975 and (571) 272-0724, respectively. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Application/Control Number: 12/970,052  
Art Unit: 1638

Page 7

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/Jeffrey R. Bolland/  
Examiner, Art Unit 1638

/Anne R. Kubelik/  
Primary Examiner, Art Unit 1638

# **Exhibit K**

• R. Rieger • A. Michaelis • M.M. Green

# Glossary of Genetics

Classical and Molecular

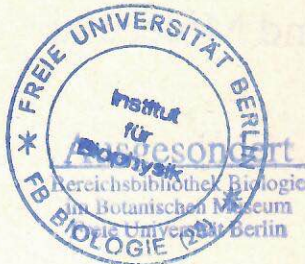
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## Preface

More than a cumstances be genetics has technology of and for the fo molecular gene this revision ha unduly increa scrupulously e either little use condensed with and redundanc the individual the specific pa related terms a

As in the p readers. We ho have been.

We are espec ticipated direct Czeschlik (Spr acknowledged.

Gatersleben an January 1991



**genomic stress**

230

G.s. combines the chemical → DNA sequencing procedure of Maxam and Gilbert with the detection of DNA sequences by → Southern blotting: Fragmented DNA is electrophoresed, transferred to an inert support, and indirectly → end-labeled by hybridization to appropriate short radioactive DNA probes.

**genomic stress** (McClintock 1978) — any of those influences which may disrupt the stability of the → genome, such as environmental factors, altered genetic background (e.g., by production of hybrids), exposition of cells to cell culture, etc. G.s. may activate silent → transposable genetic elements and result in their transposition. Reactions on g.s. are viewed as a part of the survival strategy of a cell.

**genophore** (Ris 1961) — the physical equivalent of a linkage group (the linkage structure of chromosomes) in prokaryotes, bacterial → plasmids, → mitochondria, and → chloroplasts (= genomes) which is represented by a "naked" nucleic acid molecule. The term g. was proposed to emphasize the differences in structure of → chromosomes in prokaryotes and eukaryotes.

**genospecies** (Ravin 1963) — a genetically defined → species, i.e., a group of individuals potentially able to contribute to, or share in, a common gene pool.

**genotoxic** (Brookes et al. 1973; Ehrenberg et al. 1973) — of agents (genotoxicants) inducing toxic, lethal, or heritable effects to nuclear and extranuclear genetic material in germinal and somatic cells. G. agents may be activation-independent or activation-dependent. Usually they have chemical or physical properties that facilitate their interaction with nucleic acids (→ clastogenic; mutagenic; mutagenicity testing).

At subtoxic exposure level they produce genetic alterations. In dependence on the g. agent, g. effects can be induced by different mechanisms. Direct mutagenesis results from miscopying or miscoding → DNA lesions. Indirect mutagenesis results from an error-prone replication activity after induction of noncoding DNA alterations.

**genotroph** (Durrant 1962) — a plant (in some flax varieties) showing stable heritable changes induced when it is grown in certain environments. The origination of such heritable changes is hypothetically explained by variation in the number and arrangements of particular DNA sequences.

**genotropic** (Waddington 1962) — embryonic → induction.

**genotype** (Johannsen 1909) — (1) the sum total of the → genetic information (genes) contained in the linkage structures (chromosomes) of the pro- and eukaryotes, as distinguished from their → phenotype (→ idiootype). The g. determines not a unique phenotype, but a range of phenotypic capacities referred to as an individual's "norm of reaction" to the environment. The share of one particular g. in the → gene pool of the next generation is determined by the viability and reproductive success of its carriers. (2) The genetic constitution in respect to the → alleles at one or a few → pairs of genetic loci under observation. If specific loci primarily responsible for the appearance of a particular → character are studied, the rest of the genotype is referred to as the "residual genotype or background genotype" (→ epigenotype).

**genotype-environment interaction** — that part of → phenotypic variation which is the result of interaction between genotype and environment.

**genotype frequency** — the proportion or frequency of any particular genotype among the individuals of a population. Genotype frequencies are a function of → gene frequencies. Changes in frequencies of both are brought about by systematic and/or dispersive processes.

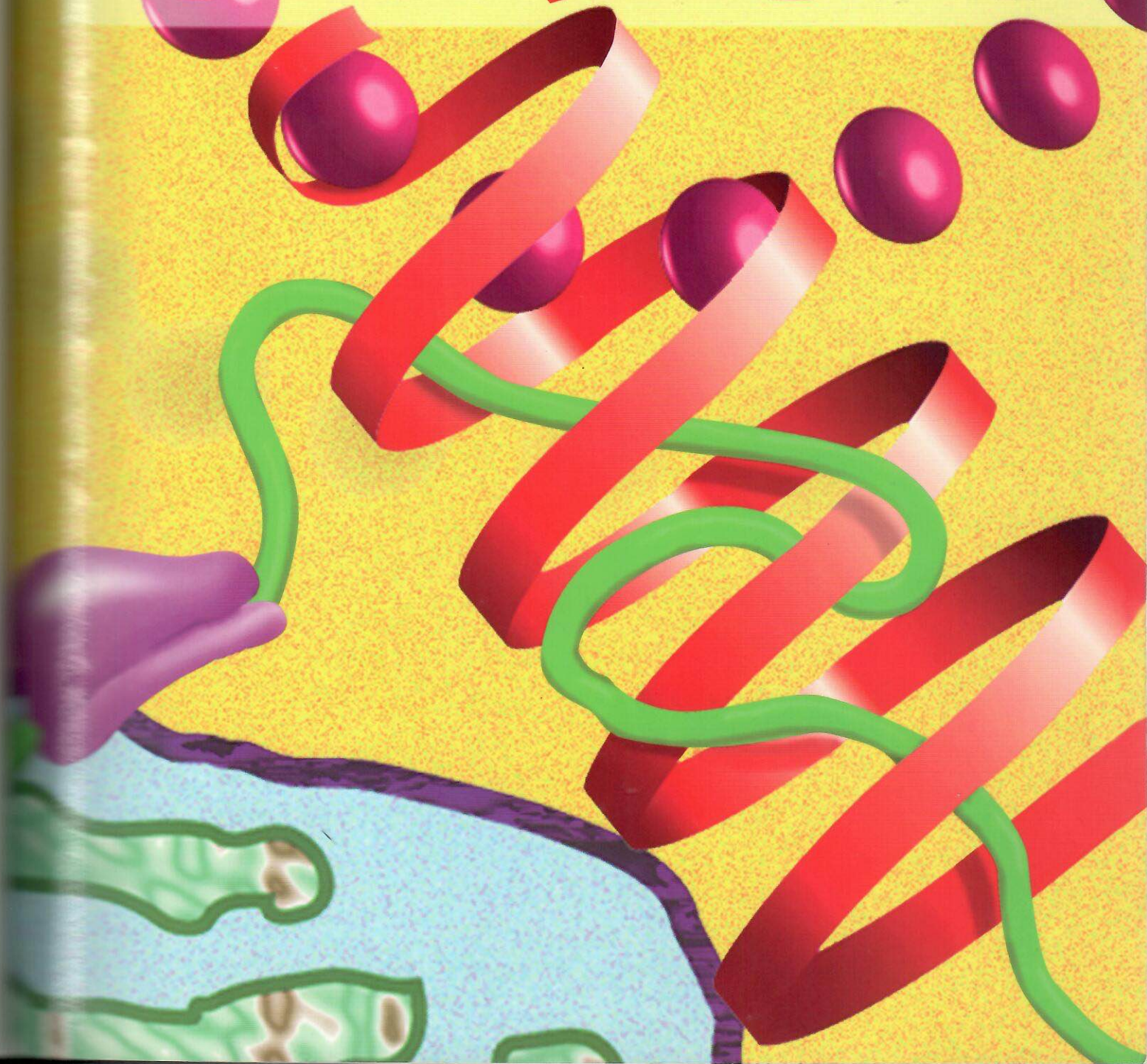
**genotypic** — ref. to any phenomenon or process connected with or controlled by the → genotype.

**genotypic cohesion** — the phenomenon wherein balanced and superior gene combinations (co-adapted gene complexes or → supergenes) are held together in the face of the centrifugal forces of → genetic recombination, thus reducing the frequency of deleterious recombinants, and with it the → genetic load.

# **Exhibit L**



# BENJAMIN LEWIN GENES V





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**Genetic marker**—*see* marker.

**Genomic (chromosomal) DNA clones** are sequences of the genome carried by a cloning vector.

**Genotype** is the genetic constitution of an organism.

**Golgi apparatus** consists of individual stacks of membranes near the endoplasmic reticulum; involved in glycosylating proteins and sorting them for transport to different cellular locations.

**G proteins** are guanine nucleotide-binding trimeric proteins that reside in the plasma membrane. When bound by GDP the trimer remains intact and is inert. When the GDP bound to the  $\alpha$  subunit is replaced by GTP, the  $\alpha$  subunit is released from the  $\beta\gamma$  dimer. One of the separated units (either the  $\alpha$  monomer or the  $\beta\gamma$  dimer) then activates or represses a target protein.

**Gratuitous inducers** resemble authentic inducers of transcription but are not substrates for the induced enzymes.

**GT-AG rule** describes the presence of these constant dinucleotides at the first two and last two positions of introns of nuclear genes.

**Gyrase** is a type II topoisomerase of *E. coli* with the ability to introduce negative supercoils into DNA.

**Hairpin** describes a double-helical region formed by base pairing between adjacent (inverted) complementary sequences in a single strand of RNA or DNA.

**Haploid set** of chromosomes contains one copy of each autosome and one sex chromosome; the haploid number  $n$  is characteristic of gametes of diploid organisms.

**Haplotype** is the particular combination of alleles in a defined region of some chromosome, in effect the genotype in miniature. Originally used to describe combinations of MHC alleles, it now may be used to describe particular combinations of RFLPs.

**Hapten** is a small molecule that acts as an antigen when conjugated to a protein.

**Helper virus** provides functions absent from a defective virus, enabling the latter to complete the infective cycle during a mixed infection.

**Hemizygote** is a diploid individual that has lost its copy of a particular gene (for example, because a chromosome has been lost) and which therefore has only a single copy.

**Heterochromatin** describes regions of the genome that are permanently in a highly condensed condition and are not genetically expressed. May be constitutive or facultative.

**Heteroduplex (hybrid) DNA** is generated by base pairing between complementary single strands derived from the different parental duplex molecules; it occurs during genetic recombination.

**Heterogametic sex** has the diploid chromosome constitution  $2A + XY$ .

**Heterogeneous nuclear (hn) RNA** comprises transcripts of nuclear genes made by RNA polymerase II; it has a wide size distribution and low stability.

**Heteromultimeric proteins** consist of nonidentical subunits (coded by different genes).

**Heterokaryon** is a cell containing two (or more) nuclei in a common cytoplasm, generated by fusing somatic cells.

**Heterozygote** is an individual with different alleles at some particular locus.

**Highly repetitive DNA** is the first component to reassociate and is equated with satellite DNA.

**Histones** are conserved DNA-binding proteins of eukaryotes that form the nucleosome, the basic subunit of chromatin.

**Homeobox** describes the conserved sequence that is part of the coding region of *D. melanogaster* homeotic genes; it is also found in amphibian and mammalian genes expressed in early embryonic development.

**Homeotic genes** are defined by mutations that convert one body part into another; for example, an insect leg may replace an antenna.

**Homogametic sex** has the diploid chromosome constitution  $2A + XX$ .

**Homologues** are chromosomes carrying the same genetic loci; a diploid cell has two copies of each homologue, one derived from each parent.

**Homomultimeric protein** consists of identical subunits.

**Homozygote** is an individual with the same

# **Exhibit M**



US 20060141495A1

(19) **United States**(12) **Patent Application Publication**  
**Wu**(10) **Pub. No.: US 2006/0141495 A1**(43) **Pub. Date: Jun. 29, 2006**(54) **POLYMORPHIC MARKERS AND METHODS  
OF GENOTYPING CORN****Publication Classification**(76) Inventor: **Kunsheng Wu**, Ballwin, MO (US)(51) **Int. Cl.*****C12Q 1/68*** (2006.01)***A01H 5/00*** (2006.01)(52) **U.S. Cl.** ..... **435/6; 800/320.1**

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**MONSANTO COMPANY****800 N. LINDBERGH BLVD.****ATTENTION: GAIL P. WUELLNER, IP****PARALEGAL, (E2NA)****ST. LOUIS, MO 63167 (US)**

(57)

**ABSTRACT**(21) Appl. No.: **11/218,305**(22) Filed: **Sep. 1, 2005****Related U.S. Application Data**(60) Provisional application No. 60/606,880, filed on Sep.  
1, 2004.

Polymorphic corn DNA loci useful for genotyping between at least two varieties of corn. Sequences of the loci are useful for designing primers and probe oligonucleotides for detecting polymorphisms in corn DNA. Polymorphisms are useful for genotyping applications in corn. The polymorphic markers are useful to establish marker/trait associations, e.g. in linkage disequilibrium mapping and association studies, positional cloning and transgenic applications, marker-aided breeding and marker-assisted selection, and identity by descent studies. The polymorphic markers are also useful in mapping libraries of DNA clones, e.g. for corn QTLs and genes linked to polymorphisms.



US 2006/0141495 A1

Jun. 29, 2006

1

## POLYMORPHIC MARKERS AND METHODS OF GENOTYPING CORN

### INCORPORATION OF SEQUENCE LISTING

[0001] Two copies of the sequence listing (Copy 1 and Copy 2) and a computer readable form (CRF) of the sequence listing, all on CD-ROMs, each containing the file named CornSNP2005.ST25.txt which is 65,830, 912 bytes (measured in MS-DOS), all of which were created on Sep. 01, 2005 are herein incorporated by reference.

### INCORPORATION OF TABLES

[0002] Two copies of table, i.e. Table 1 named as CornSNP2005\_Table1.txt, on CD-ROMs which is 10,141, 696 bytes (measured in MS-Windows), all of which were created on Aug. 18, 2004 are herein incorporated by reference.

### FIELD OF THE INVENTION

[0003] Disclosed herein are corn polymorphisms, nucleic acid molecules related to such polymorphisms and methods of using such polymorphisms and molecules, e.g. in genotyping.

### BACKGROUND

[0004] Polymorphisms are useful as genetic markers for genotyping applications in the agriculture field, e.g. in plant genetic studies and commercial breeding. See for instance U.S. Pat. Nos. 5,385,835; 5,437,697; 5,385,835; 5,492,547; 5,746,023; 5,962,764; 5,981,832 and 6,100,030, and U.S. applications Ser. No. 09/861,478 (filed May 18, 2001), Ser. No. 09/969,373 (filed Oct. 2, 2001), and Ser. No. 10/389,566 (filed Mar. 14, 2003), the disclosures of all of which are incorporated herein by reference. The highly conserved nature of DNA combined with the rare occurrences of stable polymorphisms provides genetic markers, which are both predictable and discerning of different genotypes. Among the classes of existing genetic markers are a variety of polymorphisms indicating genetic variation including restriction-fragment-length polymorphisms (RFLPs), amplified fragment-length polymorphisms (AFLPs), simple sequence repeats (SSRs), single nucleotide polymorphisms (SNPs) and insertion/deletion polymorphisms (Indels). Because the number of genetic markers for a plant species is limited, the discovery of additional genetic markers will facilitate genotyping applications including marker-trait association studies, gene mapping, gene discovery, marker-assisted selection and marker-assisted breeding. Evolving technologies make certain genetic markers more amenable for rapid, large scale use. For instance, technologies for SNP detection indicate that SNPs may be preferred genetic markers.

### SUMMARY OF THE INVENTION

[0005] This invention provides a large number of genetic markers from corn genomic DNA. These genetic markers comprise corn DNA loci, which are useful for genotyping applications involving at least two varieties of corn. A polymorphic corn locus of this invention comprises at least 20 consecutive nucleotides which include or are adjacent to a polymorphism which is identified herein, e.g. in Table 1.

[0006] One aspect of this invention is a method of analyzing DNA of a corn plant comprising the steps of obtaining a DNA sequence from a corn line for use as a query; accessing corn DNA sequences having SNP markers including DNA sequences from the Collection of Corn Marker Sequences identified in Table 1, e.g. where the set of polymorphic corn DNA sequences comprises any one of SEQ ID NO:1 through SEQ ID NO: 25043; determining the identity of said query to accessed corn DNA sequences over a window of at least 20 nucleotides; identifying accessed corn DNA sequences having a minimal identity of 90 percent to said query and identifying a SNP marker in said accessed corn DNA sequences; and using the identified SNP marker to genotype the corn line. In one aspect the method of the invention is practiced by accessing corn DNA sequences assembled and stored on a computer readable medium. In genotyping a sequence of DNA extracted from a corn plant is analyzed by comparing the extracted DNA sequence with sequences in a selected set of polymorphic DNA sequences, e.g. to identify polymorphisms in the DNA extracted from a corn plant. In one aspect of the method the selected set comprises all of the DNA sequences of SEQ ID NO: 1 through SEQ ID NO: 25043. In other aspects of the method the selected set can comprise significantly fewer of the polymorphic corn DNA sequences, e.g. a set of limited to a single chromosome or QTL or a set that is relatively evenly distributed over the genome, or a set which is informative for a trait.

[0007] Another aspect of this invention provides a method for determining the genotype of a corn plant by analyzing DNA of a corn plant, e.g. by determining the presence of a polymorphic allelic sequence in the DNA of a corn plant, its transcribed mRNA or its translated amino acids and comparing the determined sequence to the sequence of a selected set of polymorphic corn DNA sequences, their transcribed mRNA or translated amino acids. Such comparing allows the identification of allelic character of polymorphisms in the genome of a corn plant. Still another aspect of this invention provides a method for analyzing DNA of a corn plant by assaying DNA from tissue of a corn plant to identify the allelic state of a nucleic acid polymorphism in a polymorphic corn DNA locus identified herein in Table 1. Such assaying can comprise amplifying segments of corn DNA using a pair of oligonucleotide primers designed to hybridize to the 5' end of each of opposite strands of a segment of corn DNA including a polymorphism which is identified in Table 1. The assaying can further comprise hybridizing an oligonucleotide detector, e.g. having a sequence which hybridizes to the sequence of the DNA at or adjacent to the polymorphism. In such assaying the oligonucleotide primers and oligonucleotide detector can be designed to hybridize to segments of one of the selected set of DNA sequences. A useful assay includes Taqman® assays for SNP detection.

[0008] Another aspect of this invention provides a method of analyzing DNA of a corn plant further comprising identifying one or more phenotypic traits for at least two corn lines and determining associations between said traits and polymorphisms.

[0009] Still another aspect of this invention is directed to the use of a selected set of polymorphic corn DNA sequences in corn breeding, e.g. by selecting a corn line on



US 2006/0141495 A1

Jun. 29, 2006

2

the basis of its genotype at a polymorphic locus has a sequence within the selected set of polymorphic corn DNA sequences.

[0010] Yet another aspect of this invention provides a method of associating a phenotypic trait to a genotype in a population of corn plants wherein said associating comprises

[0011] (a) measuring or characterizing a set of one or more distinct phenotypic traits characterizing the corn plants,

[0012] (b) selecting tissue from at least two corn plants having polymorphic DNA and assaying DNA from the tissue to identify the allelic state of a set of distinct polymorphisms, e.g. as identified herein in Table 1, and

[0013] (c) identifying associations between the set of allelic states and the set of phenotypic traits,

where the set of polymorphisms are in loci having sequence in a subset of polymorphic corn DNA sequences. In one aspect of associating the set of polymorphisms comprises at least three, more preferably at least five or more, polymorphisms linked to mapped polymorphisms. A further aspect of the invention contemplates mapping a locus that directly affects a trait of interest by utilizing trait-marker associations discovered using SNP markers disclosed herein, e.g. where the SNP markers are linked to loci permitting disequilibrium mapping of the loci.

[0014] Still another aspect of this invention is directed to identifying genes affecting a trait of interest by identifying genes that are genetically or physically linked to a polymorphism wherein said polymorphism is associated with the trait, e.g. using markers of this invention. Such marker/trait association can be useful in marker assisted breeding. More particularly, an aspect of this invention provides a method of corn breeding comprising the steps of

[0015] (a) associating an allele of a SNP marker listed in Table 1 with a trait;

[0016] (b) genotyping corn lines using said SNP maker,

[0017] (c) selecting at least two of said genotyped corn lines which have said allele of a SNP marker;

[0018] (d) breeding said selected corn lines to produce progeny.

[0019] A further aspect of this invention provides corn plants, including plant parts such as oil, progeny seeds, protein, etc., from corn plant produced by such marker assisted breeding methods.

[0020] The methods of this invention characterized by marker identification can be carried out using oligonucleotide primers and oligonucleotides detectors. Thus, another aspect of the invention is directed to such oligonucleotides, e.g. sets of oligonucleotides functional with a marker. More particularly, this invention provides a pair of isolated nucleic acid molecules comprising oligonucleotide primers for amplifying corn DNA to identify the presence of a polymorphism in the DNA, e.g. oligonucleotides comprising at least 12 consecutive nucleotides which are at least 90% identical to ends of a segment of DNA of the same number of nucleotides in opposite strands of a polymorphic corn DNA locus having a sequence which is at least 90% identical

to a sequence in a subset of polymorphic corn DNA sequences disclosed herein (or a complement thereof). More preferably such a pair of oligonucleotides comprise at least 15 consecutive nucleotides, or more, e.g. at least 20 consecutive nucleotides. More particularly, when hybridization to a SNP is contemplated for marker assay for identifying a polymorphism in corn DNA, a set will comprise four oligonucleotides, e.g. a pair of isolated nucleic acid molecules for amplifying DNA which can hybridize to DNA which flanks a polymorphism and a pair of detector nucleic acid molecules which are useful for detecting each nucleotide in a single nucleotide polymorphism in a segment of the amplified DNA. In preferred aspects of the invention such detector nucleic acid molecules comprise at least 12 nucleotide bases and a detectable label, or at least 15 nucleotide bases, and the sequence of the detector nucleic acid molecules is identical except for the nucleotide polymorphism (e.g. SNP or Indel) and is at least 95 percent identical to a sequence of the same number of consecutive nucleotides in either strand of the segment of polymorphic corn DNA locus containing the polymorphism.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

##### A. Definitions:

As used herein certain terms are defined as follows.

[0021] An “allele” means an alternative sequence at a particular locus; the length of an allele can be as small as 1 nucleotide base, but is typically characterized by a longer sequence of common nucleotides bordering the alternative nucleotide. Allelic sequence can be amino acid sequence or nucleic acid sequence. A “locus” is a short sequence that is usually unique and usually found at one particular location in the genome by a point of reference, e.g. a short DNA sequence that is a gene, or part of a gene or intergenic region. A locus of this invention can be a unique PCR product at a particular location in the genome. The loci of this invention comprise one or more polymorphisms i.e. alternative alleles present in some individuals. “Genotype” means the specification of an allelic composition at one or more loci within an individual organism. In the case of diploid organisms, there are two alleles at each locus; a diploid genotype is said to be homozygous when the alleles are the same, and heterozygous when the alleles are different.

[0022] “Consensus sequence” means DNA sequence constructed as the consensus at each nucleotide position of a cluster of aligned sequences. Such clusters are used to identify SNP and Indel polymorphisms in alleles at a locus. Consensus sequence can be based on either strand of DNA at the locus and states the nucleotide base of either one of each SNP in the locus and the nucleotide bases of all Indels in the locus. Thus, although a consensus sequence may not be a copy of an actual DNA sequence, a consensus sequence is useful for precisely designing primers and probes for actual polymorphisms in the locus.

[0023] “Phenotype” means the detectable characteristics of a cell or organism which are a manifestation of gene expression.

[0024] “Marker” means a polymorphic sequence. A “polymorphism” is a variation among individuals in sequence, particularly in DNA sequence. Useful polymorphisms

US 2006/0141495 A1

Jun. 29, 2006

3

include single base substitutions (single nucleotide polymorphisms SNPs), or insertions or deletions in DNA sequence (Indels) and simple sequence repeats of DNA sequence (SSRs). As used herein "Collection of Corn Marker Sequences" means the set of corn DNA sequences consisting of SEQ ID NO:1 through SEQ ID NO: 25043.

[0025] As used herein "Collection of Corn SNP Markers" means the set of corn SNP markers identified in Table 2

[0026] "Marker Assay" means a method for detecting a polymorphism at a particular locus using a particular method, e.g. phenotype (such as seed color, flower color, or other visually detectable trait), restriction fragment length polymorphism (RFLP), single base extension, electrophoresis, sequence alignment, allelic specific oligonucleotide hybridization (ASO), RAPID, etc. Preferred marker assays include single base extension as disclosed in U.S. Pat. No. 6,013,431 and allelic discrimination where endonuclease activity releases a reporter dye from a hybridization probe as disclosed in U.S. Pat. No. 5,538,848 the disclosures of both of which are incorporated herein by reference.

[0027] "Linkage" refers to relative frequency at which types of gametes are produced in a cross. For example, if locus A has genes "A" or "a" and locus B has genes "B" or "b" and a cross between parent I with AABB and parent B with aabb will produce four possible gametes where the genes are segregated into AB, Ab, aB and ab. The null expectation is that there will be independent equal segregation into each of the four possible genotypes, i.e. with no linkage  $\frac{1}{4}$  of the gametes will of each genotype. Segregation of gametes into a genotypes differing from  $\frac{1}{4}$  are attributed to linkage.

[0028] "Linkage disequilibrium" is defined in the context of the relative frequency of gamete types in a population of many individuals in a single generation. If the frequency of allele A is p, a is p', B is q and b is q', then the expected frequency (with no linkage disequilibrium) of genotype AB is pq, Ab is pq', aB is p'q and ab is p'q'. Any deviation from the expected frequency is called linkage disequilibrium.

[0029] "Quantitative Trait Locus (QTL)" means a locus that controls to some degree numerically representable traits that are usually continuously distributed.

[0030] Nucleic acid molecules or fragments thereof of the present invention are capable of hybridizing to other nucleic acid molecules under certain circumstances. As used herein, two nucleic acid molecules are said to be capable of hybridizing to one another if the two molecules are capable of forming an anti-parallel, double-stranded nucleic acid structure. A nucleic acid molecule is said to be the "complement" of another nucleic acid molecule if they exhibit "complete complementarity" i.e. each nucleotide in one sequence is complementary to its base pairing partner nucleotide in another sequence. Two molecules are said to be "minimally complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under at least conventional "low-stringency" conditions. Similarly, the molecules are said to be "complementary" if they can hybridize to one another with sufficient stability to permit them to remain annealed to one another under conventional "high-stringency" conditions. Nucleic acid molecules which hybridize to other nucleic acid molecules, e.g. at least under low stringency conditions are said

to be "hybridizable cognates" of the other nucleic acid molecules. Conventional stringency conditions are described by Sambrook et al., *Molecular Cloning*, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Press, Cold Spring Harbor, New York, 1989 (Now onwards referred as Sambrook et al.) and by Haymes et al., *Nucleic Acid Hybridization, A Practical Approach*, IRL Press, Washington, DC (1985), each of which is incorporated herein by reference. Departures from complete complementarity are therefore permissible, as long as such departures do not completely preclude the capacity of the molecules to form a double-stranded structure. Thus, in order for a nucleic acid molecule to serve as a primer or probe it need only be sufficiently complementary in sequence to be able to form a stable double-stranded structure under the particular solvent and salt concentrations employed.

[0031] Appropriate stringency conditions which promote DNA hybridization, for example, 6.0x sodium chloride/sodium citrate (SSC) at about 45° C., followed by a wash of 2.0xSSC at 50° C., are known to those skilled in the art or can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6, incorporated herein by reference. For example, the salt concentration in the wash step can be selected from a low stringency of about 2.0xSSC at 50° C. to a high stringency of about 0.2xSSC at 50° C. In addition, the temperature in the wash step can be increased from low stringency conditions at room temperature, about 22° C., to high stringency conditions at about 65° C. Both temperature and salt may be varied, or either the temperature or the salt concentration may be held constant while the other variable is changed.

[0032] In a preferred embodiment, a nucleic acid molecule of the present invention will specifically hybridize to one strand of a segment of corn DNA having a nucleic acid sequence as set forth in SEQ ID NO: 1 through SEQ ID NO: 8783 under moderately stringent conditions, for example at about 2.0xSSC and about 65° C., more preferably under high stringency conditions such as 0.2xSSC and about 65° C.

[0033] As used herein "sequence identity" refers to the extent to which two optimally aligned polynucleotide or peptide sequences are invariant throughout a window of alignment of components, e.g. nucleotides or amino acids. An "identity fraction" for aligned segments of a test sequence and a reference sequence is the number of identical components which are shared by the two aligned sequences divided by the total number of components in reference sequence segment, i.e. the entire reference sequence or a smaller defined part of the reference sequence. "Percent identity" is the identity fraction times 100.

#### B. Nucleic Acid Molecules—Loci, Primers and Probes

[0034] The corn loci of this invention comprise DNA sequence, which comprises at least 20 consecutive nucleotides and includes or is adjacent to one or more polymorphisms identified in Table 1. Such corn loci have a nucleic acid sequence having at least 90% sequence identity, more preferably at least 95% or even more preferably for some alleles at least 98% and in many cases at least 99% sequence identity, to the sequence of the same number of nucleotides in either strand of a segment of corn DNA which includes or is adjacent to the polymorphism. The nucleotide sequence of one strand of such a segment of corn DNA may be found in

US 2006/0141495 A1

Jun. 29, 2006

4

a sequence in the group consisting of SEQ ID NO: 1 through SEQ ID NO: 25043. It is understood by the very nature of polymorphisms that for at least some alleles there will be no identity at the polymorphic site itself. Thus, sequence identity can be determined for sequence that is exclusive of the polymorphism sequence. The polymorphisms in each locus are identified more particularly in Table 1.

**[0035]** For many genotyping applications it is useful to employ as markers polymorphisms from more than one locus. Thus, one aspect of the invention provides a collection of different loci. The number of loci in such a collection can vary but will be a finite number, e.g. as few as 2 or 5 or 10 or 25 loci or more, for instance up to 40 or 75 or 100 or more loci, e.g. selected because they comprise a set which is limited to a single chromosome or QTL or is relatively evenly distributed over the genome, or is informative for one or more traits.

**[0036]** Another aspect of the invention provides nucleic acid molecules which are capable of hybridizing to the polymorphic corn loci of this invention. In certain embodiments of the invention, e.g. which provide PCR primers, such molecules comprises at least 15 nucleotide bases. Molecules useful as primers can hybridize under high stringency conditions to a one of the strands of a segment of DNA in a polymorphic locus of this invention. Primers for amplifying DNA are provided in pairs, i.e. a forward primer and a reverse primer. One primer will be complementary to one strand of DNA in the locus and the other primer will be complementary to the other strand of DNA in the locus, i.e. the sequence of a primer is preferably at least 90%, more preferably at least 95%, identical to a sequence of the same number of nucleotides in one of the strands. It is understood that such primers can hybridize to sequence in the locus which is distant from the polymorphism, e.g. at least 5, 10, 20, 50 or up to about 100 nucleotide bases away from the polymorphism. Design of a primer of this invention will depend on factors well known in the art, e.g. avoidance of repetitive sequence.

**[0037]** Another aspect of the nucleic acid molecules of this invention are hybridization probes for polymorphism assays. In one aspect of the invention such probes are oligonucleotides comprising at least 12 nucleotide bases and a detectable label. The purpose of such a molecule is to hybridize, e.g. under high stringency conditions, to one strand of DNA in a segment of nucleotide bases which includes or is adjacent to the polymorphism of interest in an amplified part of a polymorphic locus. Such oligonucleotides are preferably at least 90%, more preferably at least 95%, identical to the sequence of a segment of the same number of nucleotides in one strand of corn DNA in a polymorphic locus. The detectable label can be a radioactive element or a dye. In preferred aspects of the invention, the hybridization probe further comprises a fluorescent label and a quencher, e.g. for use hybridization probe assays of the type known as Taqman® assays, available from Applied Biosystems, Foster City, Calif.

**[0038]** For assays where the molecule is designed to hybridize adjacent to a polymorphism which is detected by single base extension, e.g. of a labeled dideoxynucleotide, such molecules can comprise at least 15, more preferably at least 16 or 17, nucleotide bases in a sequence which is at least 90 percent, preferably at least 95%, identical to a

sequence of the same number of consecutive nucleotides in either strand of a segment of polymorphic corn DNA. Oligonucleotides for single base extension assays are available from Orchid Biosciences, Inc.

**[0039]** Such primer and probe molecules are generally provided in groups of two primers and one or more probes for use in genotyping assays. Moreover, it is often desirable to conduct a plurality of genotyping assays for a plurality of polymorphisms. Thus, this invention also provides collections of nucleic acid molecules, e.g. in sets which characterize a plurality of polymorphisms.

#### C. Identifying Polymorphisms

**[0040]** Polymorphisms in a genome can be determined by comparing cDNA sequence from different lines. While the detection of polymorphisms by comparing cDNA sequence is relatively convenient, evaluation of cDNA sequence allows no information about the position of introns in the corresponding genomic DNA. Moreover, polymorphisms in non-coding sequence cannot be identified from cDNA. This can be a disadvantage, e.g. when using cDNA-derived polymorphisms as markers for genotyping of genomic DNA. More efficient genotyping assays can be designed if the scope of polymorphisms includes those present in non-coding unique sequence.

**[0041]** Genomic DNA sequence is more useful than cDNA for identifying and detecting polymorphisms. Polymorphisms in a genome can be determined by comparing genomic DNA sequence from different lines. However, the genomic DNA of higher eukaryotes typically contain a large fraction of repetitive sequence and transposons. Genomic DNA can be more efficiently sequenced if the coding/unique fraction is enriched by subtracting or eliminating the repetitive sequence.

**[0042]** There are a number of strategies that can be employed to enrich for coding/unique sequence. Examples of these include the use of enzymes which are sensitive to cytosine methylation, the use of the MspI endonuclease to cleave repetitive sequence, and the printing of microarrays of genomic libraries which are then hybridized with repetitive sequence probes.

**[0043]** C.1. methylated cytosine sensitive enzymes: The DNA of higher eukaryotes tends to be very heavily methylated, however it is not uniformly methylated. In fact, repetitive sequence is much more highly methylated than coding sequence. Coding/unique sequence can therefore be enriched by exploiting this difference in methylation pattern. See U.S. Pat. No. 6,017,704 for methods of mapping and assessment of DNA methylation patterns in CG islands. Some restriction endonucleases are sensitive to the presence of methylated cytosine residues in their recognition site. Such methylation sensitive restriction endonucleases may not cleave at their recognition site if the cytosine residue in either an overlapping 5'-CG-3' or an overlapping 5'-CNG-3' is methylated. Methylation sensitive restriction endonucleases include the 4 base cutters: Aci I, Hha I, HinfI, HpaII and Msp I, the 6 base cutters: Apa I, Age I, Bsr F I, BssH II, Eag I, Eae I, MspM II, Nar I, Pst I, Pvu I, Sac II, Sma I, Stu I and Xho I and the 8 base cutter: Not I. For example, DNA cleavage at the site CTGCAG by Pst I is inhibited when the C residues are methylated. In order to enrich for coding/unique sequence corn libraries can be



US 2006/0141495 A1

Jun. 29, 2006

5

constructed from genomic DNA digested with Pst I (or other methylation sensitive enzymes), and size fractionated by agarose gel electrophoresis. Regions of the genome which are heavily methylated (i.e., regions with a high fraction of repetitive sequences) have a higher number of Pst I sites that are methylated. Therefore, most of the Pst I sites in repetitive DNA will not be cleaved during Pst I digestion, and the repetitive sequence will tend to consist mostly of high molecular weight, uncleaved DNA. In contrast, regions of the genome that are not heavily methylated (i.e. regions containing a large fraction of coding/unique sequence) should contain a large fraction of unmethylated Pst I sites which will be cleaved during digestion, producing relatively smaller fragments. When digested DNA is electrophoresed through agarose, relatively larger fragments from heavily methylated, non-coding DNA regions are separated from relatively smaller fragments derived from coding/unique sequence. Coding region-enriched DNA fragments (commonly between 500-3000 bp) can be excised from the gel, purified and ligated into a Pst I digested vector, e.g. pUC18. The ligation products are transformed by electroporation into a plurality of suitable bacterial hosts, e.g. DH10B, to produce a library of clones enriched for coding/unique sequence. Individual clones can be sequenced to provide the sequence of the inserted coding region DNA.

[0044] In order to reduce the sequence complexity of any particular library, the DNA in the range 500 to 10,000 bp can be further size-fractionated by incrementally excising fragments from the gel. Useful ranges of size-fractionated fragments include 500-600 bp, 600-700 bp, 700-800 bp, 800-900 bp, 900-1100 bp, 1100-1500 bp, 1500-2000 bp, 2000-2500 bp and 2500-3000 bp. A series of size-fractionated reduced representation libraries are constructed by ligating purified DNA from each size fraction separately to the vector. A small sample of clones from each library (for example about 400 clones) is sequenced to determine the fraction of repetitive sequence present in each particular library. Comparison of reduced representation libraries prepared from a variety of different corn lines indicates that many fractions contain less than 10% repetitive sequence and some fractions contain more than 20% repetitive sequence. Preferred reduced representation libraries contain less than 20% repetitive sequence, more preferably less than 15% repetitive sequence and even more preferably less than 10% repetitive sequence. By determining the fraction of repetitive sequence throughout the whole series of size fractionated reduced representation libraries, the libraries with the smallest fraction of repetitive sequence can be selected for deep sequencing (usually 10,000-20,000 clones). Since the purpose of obtaining sequence is for polymorphism detection, the equivalent libraries representing the same size fraction for both corn strains are sequenced, or alternatively a library consisting of a mixture of DNA from different corn strains is sequenced. Another advantage of using reduced representation libraries for polymorphism detection is that it increases the probability of recovering the equivalent sequences from both corn lines. Polymorphisms can only be detected if the equivalent sequence is available from both lines.

#### C.2. McrBC endonuclease

[0045] An alternative method for enriching coding region DNA sequence enrichment uses McrBC endonuclease restriction. As a defense against invading foreign DNA from

phage/viruses, *E. coli* contain endonucleases, e.g. McrBC endonuclease, which cleave methylated cytosine-containing DNA. This feature can be exploited to enrich DNA with regions of the genome which are not heavily methylated, e.g. the presumed coding region DNA. Reduced representation libraries can be constructed using genomic DNA fragments which are cleaved by physical shearing or digestion with any restriction enzyme. DNA fragments are transformed into an *E. coli* host that contains an McrBC endonuclease, e.g. *E. coli* strain JM107 or DH5a. When the bacterial host is transformed with a DNA fragment which contains methylated DNA region, the McrBC endonuclease will cleave the inserted DNA and the plasmid will not be propagated. When the bacterial host is transformed with a DNA fragment that is not methylated, the plasmid will be propagated, and a colony will grow on the agar plate allowing the clone to be sequenced. A small sample of clones from libraries generated in this manner are sampled, and the fraction of repetitive sequenced determined. McrBC endonuclease can also be used with methylated cytosine sensitive endonuclease to further reduce the fraction of repetitive sequence in libraries that are not suitable for sequencing, e.g. sequences that contain more than 15% repetitive sequence.

#### C.3. Microarraying Reduced Representation Libraries

[0046] Another method to enrich for coding/unique sequence is to construct reduced representation libraries (using methylation sensitive or non-methylation sensitive enzymes), print microarrays of the library on nylon membrane, and hybridize with probes made from repetitive elements known to be present in the library. Clones containing repetitive sequence elements are identified, and the library is re-arrayed by picking only the negative clones. This process is performed by randomly picking clones from a reduced representation library into 384-well plates and culturing them. Micro-arrays can be prepared by printing clone DNA from the collection of 384-well plates in determined patterns on supports, such as glass supports or nylon membranes. The fabrication of microarrays comprising thousands of distinct clones, e.g. up to about 25,000 clones or more, are well known in the art. See for instance, U.S. Pat. No. 5,807,522 for methods for fabricating microarrays of spotted polynucleotides at high density. A small sample of clones from the reduced representation library, e.g. about 400 clones, can be sequenced to identify repetitive sequence elements. Clones containing the repetitive sequences are retrieved, and the clones used to make radioactive probes which are hybridized on the nylon arrays. Radioactive isotope label elements include  $^{32}\text{P}$ ,  $^{33}\text{P}$ ,  $^{35}\text{S}$ ,  $^{125}\text{I}$ , and the like with  $^{33}\text{P}$  being especially preferred. The arrays are analyzed for hybridization by detecting radiation, e.g. using a Fuji Phosphorimager™ imaging screen. After an appropriate exposure time the array image is read as a digital file representing the hybridization intensity from each array element which is proportional to amount of labeled repeat sequence. This radiation image identifies all the clones on the array which correspond to repetitive sequence clones, and also identifies the 384-well plate and well location of each repetitive sequence clone. With this information, all the non-repetitive sequence clones can be picked from the original plates and relocated onto a new set of plates which do not contain repetitive sequence clones. This method can be used to lower the fraction of repetitive sequence in reduced representation libraries from approximately 25% to about 1-2%.

US 2006/0141495 A1

Jun. 29, 2006

6

## D. Detecting Polymorphisms

[0047] Polymorphisms in DNA sequences can be detected by a variety of effective methods well known in the art including those disclosed in U.S. Pat. Nos. 5,468,613 and 5,217,863; 5,210,015; 5,876,930; 6,030,787 6,004,744; 6,013,431; 5,595,890; 5,762,876; 5,945,283; 5,468,613; 6,090,558; 5,800,944 and 5,616,464, all of which are incorporated herein by reference in their entireties. For instance, polymorphisms in DNA sequences can be detected by hybridization to allele-specific oligonucleotide (ASO) probes as disclosed in U.S. Pat. Nos. 5,468,613 and 5,217,863. The nucleotide sequence of an ASO probe is designed to form either a perfectly matched hybrid or to contain a mismatched base pair at the site of the variable nucleotide residues. The distinction between a matched and a mismatched hybrid is based on differences in the thermal stability of the hybrids in the conditions used during hybridization or washing, differences in the stability of the hybrids analyzed by denaturing gradient electrophoresis or chemical cleavage at the site of the mismatch.

[0048] U.S. Pat. No. 5,468,613 discloses allele specific oligonucleotide hybridizations where single or multiple nucleotide variations in nucleic acid sequence can be detected in nucleic acids by a process in which the sequence containing the nucleotide variation is amplified, spotted on a membrane and treated with a labeled sequence-specific oligonucleotide probe.

[0049] Length variation in DNA nucleotide sequence repeats such as microsatellites, simple sequence repeats (SSRs) and short tandem repeats (STRs) can be detected by mass spectroscopy methods as disclosed in U.S. Pat. No. 6,090,558. The advantages of using mass spectrometry include a dramatic increase in both the speed of analysis (a few seconds per sample) and the accuracy of direct mass measurements.

[0050] Target nucleic acid sequence can also be detected by probe ligation methods as disclosed in U.S. Pat. No. 5,800,944 where sequence of interest is amplified and hybridized to probes followed by ligation to detect a labeled part of the probe.

[0051] Target nucleic acid sequence can also be detected by probe linking methods as disclosed in U.S. Pat. No. 5,616,464 employing at least one pair of probes having sequences homologous to adjacent portions of the target nucleic acid sequence and having side chains which non-covalently bind to form a stem upon base pairing of said probes to said target nucleic acid sequence. At least one of the side chains has a photoactivatable group which can form a covalent cross-link with the other side chain member of the stem.

## D.1. Primer Base Extension Assay

[0052] A preferred method for detecting SNPs and Indels is a labeled base extension method as disclosed in U.S. Pat. Nos. 6,004,744; 6,013,431; 5,595,890; 5,762,876; and 5,945,283. These methods are based on primer extension and incorporation of detectable nucleoside triphosphates. The primer is designed to anneal to the sequence immediately adjacent to the variable nucleotide which can be detected after incorporation of as few as one labeled nucleoside triphosphate. The method uses three synthetic oligonucleotides. Two of the oligonucleotides serve as PCR

primers and are complementary to sequence of the locus of corn genomic DNA which flanks a region containing the polymorphism to be assayed. Using corn genomic DNA as a template the primer oligonucleotides are used in PCR to produce sufficient copies of the region of the locus containing the polymorphisms so that allelic discrimination can be conducted. Following amplification of the region of the corn genome containing the polymorphism, the PCR product is mixed with the third oligonucleotide (called an extension primer), which is designed to hybridize to the amplified DNA immediately adjacent to the polymorphism in the presence of DNA polymerase and two differentially labeled dideoxynucleosidetriphosphates. If the polymorphism is present on the template, one of the labeled dideoxynucleosidetriphosphates can be added to the primer in a single base chain extension. The allele present is then inferred by determining which of the two differential labels was added to the extension primer. Homozygous samples will result in only one of the two labeled bases being incorporated and thus only one of the two labels will be detected. Heterozygous samples have both alleles present, and will thus direct incorporation of both labels (into different molecules of the extension primer) and thus both labels will be detected.

[0053] To design primers for corn polymorphism detection by single base extension the sequence of the locus is first masked to prevent design of any of the three primers to sites that match known corn repetitive elements (e.g., transposons) or are of very low sequence complexity (di- or tri-nucleotide repeat sequences). Design of primers to such repetitive elements will result in assays of low specificity, through amplification of multiple loci or annealing of the extension primer to multiple sites.

[0054] PCR primers are preferably designed (a) to have an optimal annealing temperature for PCR in the range of 55 to 60° C., (b) to have lengths in the range of 18 to 25 bases, and (c) to produce a product in the size range 75 to 200 base pairs with the polymorphism to be assayed located at least 25 bases from the 3' end of each primer. The extension primers must be chosen to contain minimal self- or inter-primer complementarity, or the efficiency and/or specificity of the PCR reaction will be reduced.

[0055] The extension primer is designed to anneal immediately adjacent to the polymorphism, such that the 3' end of the annealed extension primer immediately abuts the polymorphic site. The extension primer can lie either to the 5' or 3' side of the polymorphism; however, if it is designed to lie on the 3' side, then the sequence of the extension primer must match the reverse complement of the sequence adjacent to the polymorphism. The extension primer must contain no self-complementarity that will enable self-annealing, or the incorporation of the labeled ddNTPs may result from self-priming of the extension primer, obscuring the results of polymorphism-directed incorporation. If the nature of the sequence adjacent to the polymorphic site makes it impossible to design an extension primer that is fully non-self-complementary, the extent of self-annealing may be limited by replacing one or two bases of the extension primer with abasic sites, as long as the abasic sites are not introduced into the three 3' most positions.

[0056] The labeled ddNTPs chosen for inclusion in the reaction are determined by the nature of the polymorphism, and whether the extension primer lies those that match the

US 2006/0141495 A1

Jun. 29, 2006

7

first base of the polymorphism. For example, in the case of an AG polymorphism, the ddNTPs would be ddATP-label(1) and ddGTP-label(2) for one strand as template or ddTTP-label(1) and ddCTP-label(2) for the other strand. Labels can be chosen from among a wide variety of chemical moieties, including affinity or immunological labels, fluorescent dyes and mass tags. In the most common embodiment of the process, affinity and immunological labels are used, followed by appropriate detection reagents. In the present example, ddATP-FITC and ddGTP-biotin might be employed, followed by incubation with anti-FITC-antibody conjugated to the enzyme horseradish peroxidase (HRP-anti-FITC), and streptavidin conjugated to the enzyme alkaline phosphatase (AP-streptavidin).

#### D.2. Labeled Probe Degradation Assay

[0057] In another preferred method for detecting polymorphisms SNPs and Indels can be detected by methods disclosed in U.S. Pat. Nos. 5,210,015; 5,876,930 and 6,030,787 in which an oligonucleotide probe having a 5' fluorescent reporter dye and a 3' quencher dye covalently linked to the 5' and 3' ends of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in the suppression of the reporter fluorescence, e.g. by Forster-type energy transfer. During PCR forward and reverse primers hybridize to a specific sequence of the target DNA flanking a polymorphism. The hybridization probe hybridizes to polymorphism-containing sequence within the amplified PCR product. In the subsequent PCR cycle DNA polymerase with 5'→3' exonuclease activity cleaves the probe and separates the reporter dye from the quencher dye resulting in increased fluorescence of the reporter. A useful assay is available from Applied Biosystems as the Taqman® assay which employs four synthetic oligonucleotides in a single reaction that concurrently amplifies the corn genomic DNA, discriminates between the alleles present, and directly provides a signal for discrimination and detection. Two of the four oligonucleotides serve as PCR primers and generate a PCR product encompassing the polymorphism to be detected. Two others are allele-specific fluorescence-resonance-energy-transfer (FRET) probes. FRET probes incorporate a fluorophore and a quencher molecule in close proximity so that the fluorescence of the fluorophore is quenched. The signal from a FRET probes is generated by degradation of the FRET oligonucleotide, so that the fluorophore is released from proximity to the quencher, and is thus able to emit light when excited at an appropriate wavelength. In the assay, two FRET probes bearing different fluorescent reporter dyes are used, where a unique dye is incorporated into an oligonucleotide that can anneal with high specificity to only one of the two alleles. Useful reporter dyes include 6-carboxy-4,7,2',7'-tetrachlorofluorescein (TET), (VIC) and 6-carboxyfluorescein phosphoramidite (FAM). A useful quencher is 6-carboxy-N,N,N',N'-tetramethylrhodamine (TAMRA). Additionally, the 3' end of each FRET probe is chemically blocked so that it can not act as a PCR primer. During the assay, corn genomic DNA is added to a buffer containing the two PCR primers and two FRET probes. Also present is a third fluorophore used as a passive reference, e.g., rhodamine X (ROX) to aid in later normalization of the relevant fluorescence values (correcting for volumetric errors in reaction assembly). Amplification of the genomic DNA is initiated. During each cycle of the PCR, the FRET probes anneal in an allele-specific manner to the template DNA molecules. Annealed (but not non-annealed)

FRET probes are degraded by TAQ DNA polymerase as the enzyme encounters the 5' end of the annealed probe, thus releasing the fluorophore from proximity to its quencher. Following the PCR reaction, the fluorescence of each of the two fluorophores, as well as that of the passive reference, is determined fluorometrically. The normalized intensity of fluorescence for each of the two dyes will be proportional to the amounts of each allele initially present in the sample, and thus the genotype of the sample can be inferred.

[0058] To design primers and probes for the assay the locus sequence is first masked to prevent design of any of the three primers to sites that match known corn repetitive elements (e.g., transposons) or are of very low sequence complexity (di- or tri-nucleotide repeat sequences). Design of primers to such repetitive elements will result in assays of low specificity, through amplification of multiple loci or annealing of the FRET probes to multiple sites.

[0059] PCR primers are designed (a) to have a length in the size range of 18 to 35 bases and matching sequences in the polymorphic locus, (b) to have a calculated melting temperature in the range of 57 to 65° C., e.g. corresponding to an optimal PCR annealing temperature of 52 to 60° C., (c) to produce a product which includes the polymorphic site and has a length in the size range of 75 to 250 base pairs. The PCR primers are preferably located on the locus so that the polymorphic site is at least one base away from the 3' end of each PCR primer. The PCR primers must not contain regions that are extensively self- or inter-complementary.

[0060] FRET probes are designed to span the sequence of the polymorphic site, preferably with the polymorphism located in the 3' most ⅓ of the oligonucleotide. In the preferred embodiment, the FRET probes will have incorporated at their 3' end a chemical moiety which, when the probe is annealed to the template DNA, binds to the minor groove of the DNA, thus enhancing the stability of the probe-template complex. The probes should have a length in the range of 12 to 20 bases, and with the 3' MGB, have a calculated melting temperature of 5 to 7° C. above that of the PCR primers. Probe design is disclosed in US Pat. Nos. 5,538,848; 6,084,102 and 6,127,121.

#### E. Construction of Genetic Linkage Maps

[0061] Genetic linkage maps can be constructed using the JoinMap version 2.0 software which is described by Stam, P. "Construction of integrated genetic linkage maps by means of a new computer package: JoinMap, *The Plant Journal*, 3: 739-744 (1993); Stam, P. and van Ooijen, J. W. "JoinMap version 2.0: Software for the calculation of genetic linkage maps (1995) CPRO-DLO, Wageningen. JoinMap implements a weighted-least squares approach to multipoint mapping in which information from all pairs of linked loci (adjacent or not) is incorporated. Linkage groups are formed using a LOD threshold of 5.0.

[0062] Alternatively genetic linkage maps can be constructed using the MAPMAKER/EXP v3.0 software described by Landers et al (Lander E. S., Green P., Abrahamson J., Barlow A., Daly M. J., Lincoln S. E., and Newburg I., *Genomics* 1: 174-181, 1987). MAPMAKER/EXP performs full multipoint linkage analysis (simultaneous estimation of all recombination fractions from the primary data) for dominant, recessive, and co-dominant (e.g. RFLP-like) markers. Public SSRs, e.g. approximately 1 every 20



US 2006/0141495 A1

Jun. 29, 2006

8

cM, can be used as frameworks prior to SNP placement on the 20 linkage groups of corn (Cregan P. B., Jarvik T., Bush L., Shoemaker R. C., Lark K. G., Kahler A. L., VanToai T. T., Lohnes D. G., Chung J., Specht J. E., Crop Sci. 39:1464-1490, 1999). MAPMAKER/EXP's "group" command can be used at LOD thresholds of 20.0, 10.0, 5.0, and 3.0 for gross linkage group assignment. Next, "order" command (LOD threshold 2.0) is used to order markers within the linkage groups. The "try" command is used to place all remaining markers onto the linkage groups. Then the "ripple" command is used to verify local order. (group", "order", "try", and "ripple" commands are described in MAPMAKER/EXP). Centimorgan distance is calculated using the Kosambi or Haldane mapping function. (Kosambi D. D., Ann Eugen. 12: 172-175, 1944; Haldane, J. B. S., J. Genet. 8:299-309, 1919.).

[0063] The ordered linkage groups, defined by soft wares JointMap v 2.0 or MAPMAKER/EXP, are arranged in Microsoft Excel in accordance to the software's output. SSR and SNP loci, cM distance (Kosambi mapping function), and genotypic scores are arranged, from top to bottom, to detect possible errors in scores (double-crossovers and misscores). After verifying genotypic scores for accuracy and consistency, the loci can be once again mapped using JointMap v 2.0 or MAPMAKER/EXP to finalize map order, cM distance, and the addition of previously unmapped loci.

[0064] Jansen discloses an alternative approach for linkage map construction based on finding a locus order to minimize the total number of recombination events (Jansen J. et al. in Theor Appl Genet. 102: 1113-1122, 2001). Under many conditions this approach yields a close approximation to a maximum-likelihood map. A map estimated by this approach agrees quite closely with the map obtained using JoinMap 2.0

#### F. Use Of Polymorphisms To Establish Marker/Trait Associations

[0065] The polymorphisms in the loci of this invention can be used in marker/trait associations which are inferred from statistical analysis of genotypes and phenotypes of the members of a population. These members may be individual organisms, e.g. corn, families of closely related individuals, inbred lines, dihaploids or other groups of closely related individuals. Such corn groups are referred to as "lines", indicating line of descent. The population may be descended from a single cross between two individuals or two lines (e.g. a mapping population) or it may consist of individuals with many lines of descent. Each individual or line is characterized by a single or average trait phenotype and by the genotypes at one or more marker loci.

[0066] Several types of statistical analysis can be used to infer marker/trait association from the phenotype/genotype data, but a basic idea is to detect markers, i.e. polymorphisms, for which alternative genotypes have significantly different average phenotypes. For example, if a given marker locus A has three alternative genotypes (AA, Aa and aa), and if those three classes of individuals have significantly different phenotypes, then one infers that locus A is associated with the trait. The significance of differences in phenotype may be tested by several types of standard statistical tests such as linear regression of marker genotypes on phenotype or analysis of variance (ANOVA). Commercially available, statistical software packages commonly

used to do this type of analysis include SAS Enterprise Miner (SAS Institute Inc., Cary, N.C.) and Splus (Insightful Corporation. Cambridge, Mass.). When many markers are tested simultaneously, an adjustment such as Bonferonni correction is made in the level of significance required to declare an association.

[0067] Often the goal of an association study is not simply to detect marker/trait associations, but to estimate the location of genes affecting the trait directly (i.e. QTLs) relative to the marker locations. In a simple approach to this goal, one makes a comparison among marker loci of the magnitude of difference among alternative genotypes or the level of significance of that difference. Trait genes are inferred to be located nearest the marker(s) that have the greatest associated genotypic difference. In a more complex analysis, such as interval mapping (Lander and Botstein, *Genetics* 121:185-199 (1989), each of many positions along the genetic map (say at 1 cM intervals) is tested for the likelihood that a QTL is located at that position. The genotype/phenotype data are used to calculate for each test position a LOD score (log of likelihood ratio). When the LOD score exceeds a critical threshold value, there is significant evidence for the location of a QTL at that position on the genetic map (which will fall between two particular marker loci).

#### F.1. Linkage Disequilibrium Mapping and Association Studies

[0068] Another approach to determining trait gene location is to analyze trait-marker associations in a population within which individuals differ at both trait and marker loci. Certain marker alleles may be associated with certain trait locus alleles in this population due to population genetic process such as the unique origin of mutations, founder events, random drift and population structure. This association is referred to as linkage disequilibrium. In linkage disequilibrium mapping, one compares the trait values of individuals with different genotypes at a marker locus. Typically, a significant trait difference indicates close proximity between marker locus and one or more trait loci. If the marker density is appropriately high and the linkage disequilibrium occurs only between very closely linked sites on a chromosome, the location of trait loci can be very precise.

[0069] A specific type of linkage disequilibrium mapping is known as association studies. This approach makes use of markers within candidate genes, which are genes that are thought to be functionally involved in development of the trait because of information such as biochemistry, physiology, transcriptional profiling and reverse genetic experiments in model organisms. In association studies, markers within candidate genes are tested for association with trait variation. If linkage disequilibrium in the study population is restricted to very closely linked sites (i.e. within a gene or between adjacent genes), a positive association provides nearly conclusive evidence that the candidate gene is a trait gene.

#### F.2. Positional Cloning and Transgenic Applications

[0070] Traditional linkage mapping typically localizes a trait gene to an interval between two genetic markers (referred to as flanking markers). When this interval is relatively small (say less than 1 Mb), it becomes feasible to precisely identify the trait gene by a positional cloning

US 2006/0141495 A1

Jun. 29, 2006

9

procedure. A high marker density is required to narrow down the interval length sufficiently. This procedure requires a library of large insert genomic clones (such as a BAC library), where the inserts are pieces (usually 100-150 kb in length) of genomic DNA from the species of interest. The library is screened by probe hybridization or PCR to identify clones that contain the flanking marker sequences. Then a series of partially overlapping clones that connects the two flanking clones (a "contig") is built up through physical mapping procedures. These procedures include fingerprinting, STS content mapping and sequence-tagged connector methodologies. Once the physical contig is constructed and sequenced, the sequence is searched for all transcriptional units. The transcriptional unit that corresponds to the trait gene can be determined by comparing sequences between mutant and wild type strains, by additional fine-scale genetic mapping, and/or by functional testing through plant transformation. Trait genes identified in this way become leads for transgenic product development. Similarly, trait genes identified by association studies with candidate genes become leads for transgenic product development.

### F.3. Marker-Aided Breeding and Marker-Assisted Selection

**[0071]** When a trait gene has been localized in the vicinity of genetic markers, those markers can be used to select for improved values of the trait without the need for phenotypic analysis at each cycle of selection. In marker aided breeding and marker-assisted selection, associations between trait genes and markers are established initially through genetic mapping analysis (as in A.1 or A.2). In the same process, one determines which marker alleles are linked to favorable trait gene alleles. Subsequently, marker alleles associated with favorable trait gene alleles are selected in the population. This procedure will improve the value of the trait provided that there is sufficiently close linkage between markers and trait genes. The degree of linkage required depends upon the number of generations of selection because, at each generation, there is opportunity for breakdown of the association through recombination.

### Prediction of Crosses for New Inbred Line Development

**[0072]** The associations between specific marker alleles and favorable trait gene alleles also can be used to predict what types of progeny may segregate from a given cross. This prediction may allow selection of appropriate parents to generation populations from which new combinations of favorable trait gene alleles are assembled to produce a new inbred line. For example, if line A has marker alleles previously known to be associated with favorable trait alleles at loci 1, 20 and 31, while line B has marker alleles associated with favorable effects at loci 15, 27 and 29, then a new line could be developed by crossing A x B and selecting progeny that have favorable alleles at all 6 trait loci.

### F.4. Fingerprinting and Introgression of Transgenes

**[0073]** A fingerprint of an inbred line is the combination of alleles at a set of marker loci. High density fingerprints can be used to establish and trace the identity of germplasm, which has utility in germplasm ownership protection.

**[0074]** Genetic markers are used to accelerate introgression of transgenes into new genetic backgrounds (i.e. into a diverse range of germplasm). Simple introgression involves crossing a transgenic line to an elite inbred line and then

backcrossing the hybrid repeatedly to the elite (recurrent) parent, while selecting for maintenance of the transgene. Over multiple backcross generations, the genetic background of the original transgenic line is replaced gradually by the genetic background of the elite inbred through recombination and segregation. This process can be accelerated by selection on marker alleles that derive from the recurrent parent.

### G. Use of Polymorphism Assay for Identifying Gene of Interest.

**[0075]** The polymorphisms and loci of this invention are useful for identifying and mapping DNA sequence of QTLs and genes linked to the polymorphisms. For instance, BAC or YAC clone libraries can be queried using polymorphisms linked to a trait to find a clone containing specific QTLs and genes associated with the trait. For instance, QTLs and genes in a plurality, e.g. hundreds or thousands, of large, multi-gene sequences can be identified by hybridization with an oligonucleotide probe which hybridizes to a mapped and/or linked polymorphism. Such hybridization screening can be improved by providing clone sequence in a high density array. The screening method is more preferably enhanced by employing a pooling strategy to significantly reduce the number of hybridizations required to identify a clone containing the polymorphism. When the polymorphisms are mapped, the screening effectively maps the clones.

**[0076]** For instance, in a case where thousands of clones are arranged in a defined array, e.g. in 96 well plates, the plates can be arbitrarily arranged in three-dimensionally, arrayed stacks of wells each comprising a unique DNA clone. The wells in each stack can be represented as discrete elements in a three dimensional array of rows, columns and plates. In one aspect of the invention the number of stacks and plates in a stack are about equal to minimize the number of assays. The stacks of plates allow the construction of pools of cloned DNA.

**[0077]** For a three-dimensionally arrayed stack pools of cloned DNA can be created for (a) all of the elements in each row, (b) all of the elements of each column, and (c) all of the elements of each plate. Hybridization screening of the pools with an oligonucleotide probe which hybridizes to a polymorphism unique to one of the clones will provide a positive indication for one column pool, one row pool and one plate pool, thereby indicating the well element containing the target clone.

**[0078]** In the case of multiple stacks, additional pools of all of the clone DNA in each stack allows indication of the stack having the row-column-plate coordinates of the target clone. For instance, a 4608 clone set can be disposed in 48 96-well plates. The 48 plates can be arranged in 8 sets of 6 plate stacks providing 6x12x8 three-dimensional arrays of elements, i.e. each stack comprises 6 stacks of 8 rows and 12 columns. For the entire clone set there are 36 pools, i.e. 6 stack pools, 8 row pools, 12 column pools and 8 stack pools. Thus, a maximum of 36 hybridization reactions is required to find the clone harboring QTLs or genes associated or linked to each mapped polymorphism.

**[0079]** Once a clone is identified, oligonucleotide primers designed from the locus of the polymorphism can be used for positional cloning of the linked QTL and/or genes.

US 2006/0141495 A1

Jun. 29, 2006

10

## H. Computer Readable Media, Databases and Methods

[0080] The sequences of nucleic acid molecules of this invention can be “provided” in a variety of mediums to facilitate use, e.g. a database or computer readable medium, which can also contain descriptive annotations in a form that allows a skilled artisan to examine or query the sequences and obtain useful information. In one embodiment of the invention computer readable media may be prepared that comprise nucleic acid sequences where at least 10% or more, e.g. at least 25%, or even at least 50% or more of the sequences of the loci and nucleic acid molecules of this invention. For instance, such database or computer readable medium may comprise sets of the loci of this invention or sets of primers and probes useful for assaying the polymorphisms of this invention. In addition such database or computer readable medium may comprise a figure or table of the mapped or unmapped polymorphisms or this invention and genetic maps.

[0081] As used herein “database” refers to any representation of retrievable collected data including computer files such as text files, database files, spreadsheet files and image files, printed tabulations and graphical representations and combinations of digital and image data collections. In a preferred aspect of the invention, “database” means a memory system that can store computer searchable information. Currently, preferred database applications include those provided by DB2, Sybase and Oracle.

[0082] As used herein, “computer readable media” refers to any medium that can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc, storage medium and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. A skilled artisan can readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising computer readable medium having recorded thereon a nucleotide sequence of the present invention.

[0083] As used herein, “recorded” refers to the result of a process for storing information in a retrievable database or computer readable medium. For instance, a skilled artisan can readily adopt any of the presently known methods for recording information on computer readable medium to generate media comprising the mapped polymorphisms and other nucleotide sequence information of the present invention. A variety of data storage structures are available to a skilled artisan for creating a computer readable medium where the choice of the data storage structure will generally be based on the means chosen to access the stored information. In addition, a variety of data processor programs and formats can be used to store the polymorphisms and nucleotide sequence information of the present invention on computer readable medium.

[0084] Computer software is publicly available which allows a skilled artisan to access sequence information provided in a computer readable medium. The examples which follow demonstrate how software which implements a search algorithm such as the BLAST algorithm (Altschul et al., J. Mol. Biol. 215:403-410 (1990), incorporated herein by reference) and the BLAZE algorithm (Brutlag et al., Comp. Chem. 17:203-207 (1993), incorporated herein by

reference) on a Sybase system can be used to identify DNA sequence which is homologous to the sequence of loci of this invention with a high level of identity. Sequence of high identity can be compared to find polymorphic markers useful with corn varieties.

[0085] The present invention further provides systems, particularly computer-based systems, which contain the sequence information described herein. Such systems are designed to identify commercially important sequence segments of the nucleic acid molecules of this invention. As used herein, “a computer-based system refers to the hardware, software and memory used to analyze the nucleotide sequence information. A skilled artisan can readily appreciate that any one of many currently available computer-based systems are suitable for use in practicing the present invention by a computer-based method.

[0086] As indicated above, it is preferable to practice the methods of this invention using computer-based systems comprising a database having stored therein polymorphic markers, genetic maps, and/or the sequence of nucleic acid molecules of the present invention and the necessary hardware and software for supporting and implementing genotyping applications.

## EXAMPLE 1

[0087] This example illustrates identification of SNP and Indel polymorphisms by comparing alignments of the sequences of contigs and singletons from at least two separate maize lines. Genomic and cDNA libraries from multiple maize lines were made by isolating genomic DNA or mRNA from different maize lines by Plant DNazol Reagent or RNazol<sup>™</sup> from Life Technologies now Invitrogen (Invitrogen Life Technologies, Carlsbad, Calif.). For genomic libraries, genomic DNA were digested with Pst 1 endonuclease restriction enzyme, size fractionated over 1% agarose gel and ligated in plasmid vector for sequencing by standard molecular biology techniques as described in Sambrook et al. cDNA libraries were made by using “SuperScript<sup>™</sup> plasmid system for cDNA synthesis and plasmid cloning” kits from Life Technologies now Invitrogen (Invitrogen Life Technologies, Carlsbad, Calif.) by following manufacturers’ instructions. These libraries were sequenced by standard procedures on ABI Prism<sup>®</sup> 377 DNA Sequencer using commercially available reagents (Applied Biosystems, Foster City, Calif.). All sequences are assembled to identify non redundant sequences by Pangea Clustering and Alignment Tools which is available from DoubleTwist Inc., Oakland, Calif. Difference in sequences from multiple clones on assemblies contigs is identified as single or multiple nucleotide polymorphism. Sequence from multiple maize lines is assembled to into loci having one or more polymorphisms, i.e. SNPs and/or Indels. Candidate polymorphisms are qualified by the following parameters:

[0088] (a) The minimum length of a contig or singleton for a consensus alignment is 200 bases.

[0089] (b) The percentage identity of observed bases in a region of 15 bases on each side of a candidate SNP, is at least 75%.

[0090] (c) The minimum sequence reads in a given contig is 4.

[0091] A plurality of loci having qualified polymorphisms are identified as having consensus sequence as reported as

US 2006/0141495 A1

Jun. 29, 2006

11

SEQ ID NO: 1 through SEQ ID NO:25043. Qualified SNP and Indel polymorphisms in each locus are identified in Table 1. More particularly, Table 1 identifies the type and location of the polymorphisms as follows:

[0092] SEQ ID NO: refers to the sequence number of the polymorphic maize DNA locus of the invention, e.g. a SEQ ID NO.

[0093] SEQUENCE NAME refers to an arbitrary name for identifying the polymorphic maize DNA locus.

[0094] LENGTH refers to the length of the consensus sequence.

[0095] SNP\_ID refers to an arbitrary name for identifying each polymorphism.

[0096] POSITION refers to the position in the nucleotide sequence of the polymorphic maize DNA locus where the polymorphism occurs.

[0097] "A" refers to the total counts of sequence reads in the contig that contain an "A" at the position specified at "POSITION" column. A also refers to nucleoside Adenosine.

[0098] "C" refers to the total counts of sequence reads in the contig that contain a "C" at the position specified at "POSITION" column. C also refers to nucleoside Cytosine.

[0099] "G" refers to the total counts of sequence reads in the contig that contain a "G" at the position specified at "POSITION" column. G also refers to nucleoside Guanosine.

[0100] "T" refers to the total counts of sequence reads in the contig that contain a "T" at the position specified at "POSITION" column. T also refers to nucleoside Thymidine.

[0101] "-" refers to the total counts of sequence reads in the contig that contain a missing base or nucleoside at the position specified at "POSITION" column.

## EXAMPLE 2

[0102] This example illustrates the use of primer base extension for detecting a SNP polymorphism. Reference is made ZmSNP2004\_11516\_2\_c3430, in the polymorphic maize locus of SEQ ID NO: 969. Three polymorphisms in that locus are described more particularly in the following Table 2A which is extracted from Table 1.

TABLE 2A

SEQ ID NO:	SNP_ID	START Position	END Position	TYPE	ALLELE 1/ STRAIN 1	ALLELE 2/ STRAIN 2
969	ZmSNP2004_11516_2_c3430	3430	3430	SNP	C/MO17	G/B73
969	ZmSNP2004_11516_2_a3535	3535	3535	SNP	A/MO17	G/B73
969	ZmSNP2004_11516_2_a3784	3784	3784	SNP	A/MO17	G/B73

[0103]

TABLE 2B

Description Name	Probe	SNPSequence
PCR primer 969-3430 F	GGTTTGATCTTCTGCTT	TGGA

TABLE 2B-continued

Description Name	Probe	SNPSequence
PCR primer 969-3430 R		CACCAAACATATTGAATACTGGCTTT
SNP probe 969-3430V	VIC	C ATACGCCTTCGCTCA
SNP probe 969-3430 M	FAM	G TACGCCTTCGCTCA

[0104] With reference to Table 2B, forward and reverse PCR primers ("969-3430F" and "969-3430R") and reporter dye-tagged probes ("969-3430V" and "969-3430M") are designed to hybridize to template DNA sequence in the polymorphic maize DNA locus of SEQ ID NO: 969 around the C/G SNP polymorphism of SNP\_ID: ZmSNP2004\_11516\_2\_c3430. Such probes can be designed and provided by Applied Biosystems for their proprietary Taqman® assay (Applied Biosystems, Foster City, Calif.).

[0105] A quantity of maize genomic template DNA (e.g. about 2-20 nanograms) is mixed in 5 microliter total volume with four oligonucleotides, i.e. "969-3430F" forward primer, "969-3430R" reverse primer, "969-3430V" SNP hybridization probe having a VIC reporter attached to the 5' end, and "969-3430M" SNP hybridization probe having a FAM reporter attached to the 5' end with appropriate amount of PCR reaction buffer containing the passive reference dye ROX. The PCR reaction is conducted for 35 cycles using a 60° C. annealing-extension temperature. Following the reaction, the fluorescence of each fluorophore as well as that of the passive reference is determined in a fluorimeter. The fluorescence value for each fluorophore is normalized to the fluorescence value of the passive reference. The normalized values are plotted against each other for each sample to produce an allelogram. A successful genotyping assay using the primers and hybridization probes of this example provides an allelogram with data points in clearly separable clusters.

[0106] To confirm that an assay produces accurate results, each new assay is performed on a number of replicates of samples of known genotypic identity representing each of the three possible genotypes, i.e. two homozygous alleles

and a heterozygous sample. To be a valid and useful assay, it must produce clearly separable clusters of data points, such that one of the three genotypes can be assigned for at least 90% of the data points, and the assignment is observed to be correct for at least 98% of the data points. Subsequent to this validation step, the assay is applied to progeny of a cross between two highly inbred individuals to obtain segregation data, which are then used to calculate a genetic map position for the polymorphic locus.



US 2006/0141495 A1

Jun. 29, 2006

12

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SEQUENCE LISTING

The patent application contains a lengthy "Sequence Listing" section. A copy of the "Sequence Listing" is available in electronic form from the USPTO web site (<http://seqdata.uspto.gov/?pageRequest=docDetail&DocID=US20060141495A1>). An electronic copy of the "Sequence Listing" will also be available from the USPTO upon request and payment of the fee set forth in 37 CFR 1.19(b)(3).

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What is claimed is:

**1-18.** (canceled)

**19.** A set of four oligonucleotides useful for identifying a polymorphism in corn DNA identified in Table 1 comprising

- (a) a pair of isolated nucleic acid molecules according to claim 16 which can hybridize to DNA which flanks a polymorphism identified in Table 1;
- (b) a pair of detector nucleic acid molecules which are useful for detecting each nucleotide in a single nucleotide polymorphism in a segment of DNA amplified by said pair of nucleic acid molecule primers of (a), wherein said detector nucleic acid molecules comprise

(1) at least 12 nucleotide bases and a detectable label, or

(2) at least 15 nucleotide bases, and wherein the sequence of said detector nucleic acid molecules is identical except for said nucleotide polymorphism and is at least 95 percent identical to a sequence of the same number of consecutive nucleotides in either strand of said segment of polymorphic corn DNA locus said polymorphism.

\* \* \* \* \*

# **Exhibit N**





UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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11/938,359

11/12/2007

James Wayne Bing

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29122

7590

07/09/2010

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EXAMINER

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ART UNIT

PAPER NUMBER

1638

MAIL DATE

DELIVERY MODE

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PAPER

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

# 7530

**Office Action Summary**

Application No.

11/938,359

Applicant(s)

BING ET AL.

Examiner

Anne R. Kubelik

Art Unit

1638

**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --****Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

- 1) ☒ Responsive to communication(s) filed on 07 May 2010.
- 2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims**

- 4) ☒ Claim(s) 2 and 4-15 is/are pending in the application.
- 4a) Of the above claim(s) \_\_\_\_\_ is/are withdrawn from consideration.
- 5) ☐ Claim(s) \_\_\_\_\_ is/are allowed.
- 6) ☒ Claim(s) 2 and 4-15 is/are rejected.
- 7) ☐ Claim(s) \_\_\_\_\_ is/are objected to.
- 8) ☐ Claim(s) \_\_\_\_\_ are subject to restriction and/or election requirement.

**Application Papers**

- 9) ☐ The specification is objected to by the Examiner.
- 10) ☐ The drawing(s) filed on \_\_\_\_\_ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

**Priority under 35 U.S.C. § 119**

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some \* c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

- |   |   |
|---|---|
| 1) <input type="checkbox"/> Notice of References Cited (PTO-892)                    | 4) <input type="checkbox"/> Interview Summary (PTO-413)           |
| 2) <input type="checkbox"/> Notice of Draftperson's Patent Drawing Review (PTO-948) | Paper No(s)/Mail Date. _____                                      |
| 3) <input type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)         | 5) <input type="checkbox"/> Notice of Informal Patent Application |
| Paper No(s)/Mail Date _____   | 6) <input type="checkbox"/> Other: _____                          |

Application/Control Number: 11/938,359  
Art Unit: 1638

Page 2

### **DETAILED ACTION**

1. Claims 2 and 4-15 are pending.
2. The rejection of claims 1-3 under 35 U.S.C. 103(a) as being unpatentable over Narva et al (2002, US Patent 6,372,480) in view of Boets et al (US Patent Application Publication 2002/0199215), taken with the evidence of Crickmore et al (2009, [http://www.lifesci.sussex.ac.uk/Home/Neil\\_Crickmore/Bt/](http://www.lifesci.sussex.ac.uk/Home/Neil_Crickmore/Bt/)) is withdrawn in light of Applicant's amendment of the claims.

### ***Claim Rejections - 35 USC § 112***

3. The following is a quotation of the first paragraph of 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

4. Claims 2 and 4-15 are rejected under 35 USC 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The claims are drawn to corn plants comprising SEQ ID NO:19, a DNA construct comprising a first expression cassette comprising in operable linkage a maize ubiquitin promoter, a 5' untranslated exon of a maize ubiquitin gene, a maize ubiquitin first intron, a Cry34Ab1 encoding DNA molecule, and a PinII transcriptional terminator; a second expression cassette comprising in operable linkage a wheat peroxidase promoter, a Cry35Ab1 encoding DNA molecule, and a PinII transcriptional terminator; and a third expression cassette comprising in operable linkage aCaMV 35S promoter; a pat encoding DNA molecule; and a 3' transcriptional

Application/Control Number: 11/938,359  
Art Unit: 1638

Page 3

terminator from CaMV 35S, and SEQ ID NO:20. SEQ ID NO:19 and 20 are flanking sequences of corn event DAS-59122-7.

The only such corn plants taught in the specification are event DAS-59122-7 plants.

Since event DAS-59122-7 plants are essential to the claimed invention, they must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If a seed comprising event DAS-59122-7 is not so obtainable or available, a deposit thereof may satisfy the requirements of 35 U.S.C. 112. The specification does not disclose a repeatable process to obtain the exact same seed in each occurrence and it is not apparent if such a seed is readily available to the public.

If the deposit of these seeds is made under the terms of the Budapest Treaty, then an affidavit or declaration by the Applicant, or a statement by an attorney of record over his or her signature and registration number, stating that the seeds will be irrevocably and without restriction or condition released to the public upon the issuance of a patent would satisfy the deposit requirement made herein. A minimum deposit of 2500 seeds is considered sufficient in the ordinary case to assure availability through the period for which a deposit must be maintained.

If the deposit has not been made under the Budapest Treaty, then in order to certify that the deposit, meets the criteria set forth in 37 CFR 1.801-1.809, Applicant may provide assurance of compliance by an affidavit or declaration, or by a statement by an attorney of record over his or her signature and registration number showing that

(a) during the pendency of the application, access to the invention will be afforded to the Commissioner upon request;

Application/Control Number: 11/938,359  
Art Unit: 1638

Page 4

(b) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent;

(c) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the enforceable life of the patent, whichever is longer;

(d) the viability of the biological material at the time of deposit will be tested (see 37 CFR 1.807); and

(e) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CFR 1.809(d) should be added to the specification. See 37 CFR 1.801 - 1.809 [MPEP 2401-2411.05] for additional explanation of these requirements.

### ***Double Patenting***

5. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

Application/Control Number: 11/938,359  
Art Unit: 1638

Page 5

6. Claim 2 and 4-15 are rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 1 of U.S. Patent No. 7,323,556. Although the conflicting claims are not identical, they are not patentably distinct from each other.

Applicant's amendment of the claims is such that the claimed invention reads on the invention I in the restriction mailed 17 September 2007 in parent application 11/237,22, now U.S. Patent No. 7,323,556; this is the invention elected in that application.

SEQ ID NO:23, claimed in the issued patent, makes obvious corn plants comprising it, as claimed in the instant application, because SEQ ID NO:23 comprises SEQ ID NO:19, a DNA construct comprising a first expression cassette comprising in operable linkage a maize ubiquitin promoter, a 5' untranslated exon of a maize ubiquitin gene, a maize ubiquitin first intron, a Cry34Ab1 encoding DNA molecule, and a PinII transcriptional terminator; a second expression cassette comprising in operable linkage a wheat peroxidase promoter, a Cry35Ab1 encoding DNA molecule, and a PinII transcriptional terminator; and a third expression cassette comprising in operable linkage a CaMV 35S promoter; a pat encoding DNA molecule; and a 3' transcriptional terminator from CaMV 35S, and SEQ ID NO:20. As SEQ ID NO:19 and 20, and thus SEQ ID NO:23, are derived from the insertion of the DNA construct into a specific site in a corn plant genome, these nucleic acids make obvious corn plants comprising them.

### *Conclusion*

7. Applicant's amendment necessitated the new ground(s) of rejection presented in this Office action. Accordingly, **THIS ACTION IS MADE FINAL**. See MPEP § 706.07(a). Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO



Application/Control Number: 11/938,359

Page 6

Art Unit: 1638

MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the date of this final action.

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to Anne R. Kubelik, Ph.D., whose telephone number is (571) 272-0801. The examiner can normally be reached Monday through Friday, 8:30 am - 5:00 pm.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anne Marie Grunberg, can be reached at (571) 272-0975.

The central fax number for official correspondence is (571) 273-8300.

Patent applicants with problems or questions regarding electronic images that can be viewed in the Patent Application Information Retrieval system (PAIR) can now contact the USPTO's Patent Electronic Business Center (Patent EBC) for assistance. Representatives are available to answer your questions daily from 6 am to midnight (EST). The toll free number is (866) 217-9197. When calling please have your application serial or patent number, the type of document you are having an image problem with, the number of pages and the specific nature of the problem. The Patent Electronic Business Center will notify applicants of the resolution of the problem within 5-7 business days. Applicants can also check PAIR to confirm that the problem has been corrected. The USPTO's Patent Electronic Business Center is a complete service center supporting all patent business on the Internet. The USPTO's PAIR system provides Internet-based access to patent application status and history information. It also enables applicants to view the scanned images of their own application file folder(s) as well as general patent information available to the public.

For all other customer support, please call the USPTO Call Center (UCC) at 800-786-9199.

July 10, 2010

/Anne R Kubelik/

Primary Examiner, Art Unit 1638

# **Exhibit O**

Request for *Ex Parte* Reexamination, U.S. Patent No. 8,283,522

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

U.S. Patent No. 8,283,522	§	Attorney Docket No.: 118525-0001-001
	§	Customer No.: 28120
Issue Date: October 9, 2012	§	
	§	
Filing Date: November 3, 2008	§	
	§	
For: NOVEL HERBICIDE	§	
RESITANCE GENES	§	
	§	
	§	

**REQUEST FOR *EX PARTE* REEXAMINATION OF  
U.S. PATENT NO. 8,283,522**

Mail Stop *Ex Parte* Reexam  
Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

Dear Commissioner:

Pursuant to 35 U.S.C. § 302 and 37 C.F.R. § 1.510 et seq., the undersigned, on behalf of Inari Agriculture Inc. (the “Requester”) hereby requests *ex parte* reexamination of claims 1-3 (the “Challenged Claims”) of U.S. Patent No. 8,283,522 (“the ’522 patent,” Ex. 1001).

On its face, the ’522 patent is assigned to Dow AgroSciences LLC. However, currently, the ’522 patent is assigned to Corteva Agriscience LLC (“Corteva”). The assignment to Corteva is recorded in the U.S. Patent and Trademark Office (“USPTO”) at reel/frame 058044/0184.

**Prior and Concurrent Proceedings Involving the ’522 Patent**

The ’522 patent is not part of an active or expired litigation proceeding. The ’522 patent is also not part of any current or expired proceedings before the USPTO.

**Ex Parte Patent Reexamination Filing Requirements**

Pursuant to 37 C.F.R. § 1.510(b)(1), statements pointing out at least one substantial new question of patentability based on material, non-cumulative reference patents and printed publications for the Challenged Claims of the '522 patent are provided in Section III of this Request. Particularly, the issue of obviousness-type double patenting over the claims of U.S. Patent No. 8,916,752 ("752 patent") (Ex. 1003) raised herein was not raised in the original prosecution proceedings concerning the '522 patent.

Pursuant to 37 C.F.R. § 1.510(b)(2), reexamination of the Challenged Claims of the '522 patent is requested, and a detailed explanation of the pertinence and manner of applying the cited references to the Challenged Claims is provided in Section II of this Request.

Pursuant to 37 C.F.R. § 1.510(b)(3), copies of every patent or printed publication relied upon or referred to in the statement pointing out each substantial new question of patentability or in the detailed explanation of the pertinence and manner of applying the cited references are provided as Exhibits 1001-1004 of this Request.

Pursuant to 37 C.F.R. § 1.510(b)(4), a copy of the '522 patent is provided as Exhibit 1001 of this Request, along with a copy of any disclaimer, certificate of correction, and reexamination certificate issued corresponding to the patent.

Pursuant to 37 C.F.R. § 1.510(b)(5), the attached Certificate of Service indicates that a copy of this Request, in its entirety, has been served on Patent Owner at the following address of record for Patent Owner, in accordance with 37 C.F.R. § 1.33(c):

83067 - Faegre Drinker Biddle & Reath LLP - Dow AgroSciences  
300 North Meridian Street  
Suite 2500  
Indianapolis, IN 46204

Also submitted herewith is the fee set forth in 37 C.F.R. § 1.20(c)(1).

Pursuant to 37 C.F.R. § 1.510(b)(6), Requester hereby certifies that the statutory estoppel provisions of 35 U.S.C. § 315(e)(1) and 35 U.S.C. § 325(e)(1) do not prohibit Requester from filing this *ex parte* patent reexamination request.

## TABLE OF CONTENTS

I.	BACKGROUND OF THE REQUEST .....	1
II.	SUBSTANTIAL NEW QUESTIONS OF PATENTABILITY .....	5
A.	Listing of Prior Art Patents and Printed Publications .....	5
B.	Statement Setting Forth Each Substantial New Question of Patentability .....	6
C.	Background and Prosecution of the '522 Patent .....	7
1.	The '522 Patent .....	7
2.	Prosecution History of the '522 Patent .....	8
III.	DETAILED EXPLANATION OF THE PERTINENCE AND MANNER OF APPLYING THE PRIOR ART REFERENCES TO EVERY CLAIM FOR WHICH REEXAMINATION IS REQUESTED .....	8
A.	The Level of Ordinary Skill in the Pertinent Art .....	9
B.	Requirements for Obviousness-Type Double Patenting .....	9
1.	Legal Standards .....	9
2.	The One-Way Obviousness-Type Double Patenting Test Applies .....	14
3.	Claim Construction .....	15
C.	The Challenged Claims 1, 2 and 3 of the '522 Patent Are Unpatentable Due To Obviousness-Type Double Patenting Over '752 Claims 1, 16 and 18 .....	15
1.	'522 Claim 1 Obvious over '752 Claim 1 .....	15
2.	'522 Claim 2 Obvious over '752 Claim 16 .....	18
3.	'522 Claim 3 Obvious over '752 Claim 18 .....	19
IV.	SECONDARY CONSIDERATIONS .....	21
V.	CONCLUSION .....	22



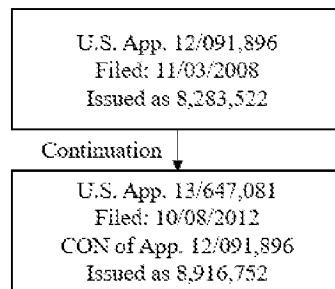
Request for *Ex Parte* Reexamination, U.S. Patent No. 8,283,522

### TABLE OF EXHIBITS

Exhibit ("Ex. ")	Description
1001	U.S. Patent No. 8,283,522 ("522 patent" or "522")
1002	File History of U.S. Patent No. 8,283,522 ("522 File History")
1003	U.S. Patent No. 8,916,752 ("752 patent" or "752")
1004	File History of U.S. Patent No. 8,916,752 ("752 File History")
1005	[Reserved]
1006	[Reserved]
1007	<i>Ex parte Collect LLC</i> , Appeal 2021-004967, Reexamination Control 90/014,452 (PTAB Dec. 1, 2021)
1008	<i>Ex parte Collect LLC</i> , Appeal 2021-005303, Reexamination Control 90/014,453 (PTAB Dec. 1, 2021)
1009	<i>Ex parte Collect LLC</i> , Appeal 2021-005302, Reexamination Control 90/014,454 (PTAB Dec. 1, 2021)
1010	<i>Ex parte Collect LLC</i> , Appeal 2021-005046, Reexamination Control 90/014,455 (PTAB Dec. 1, 2021)
1011	<i>Ex parte Collect LLC</i> , Appeal 2021-005258, Reexamination Control 90/014,457 (PTAB Dec. 1, 2021)
1012	Request for <i>Ex parte</i> Reexamination of U.S. Patent No. 6,982,740, Reexamination Control 90/014,452
1013	Request for <i>Ex parte</i> Reexamination of U.S. Patent No. 6,982,742, Reexamination Control 90/014,453
1014	Request for <i>Ex parte</i> Reexamination of U.S. Patent No. 6,424,369, Reexamination Control 90/014,454
1015	Request for <i>Ex parte</i> Reexamination of U.S. Patent No. 6,452,626, Reexamination Control 90/014,455
1016	Request for <i>Ex parte</i> Reexamination of U.S. Patent No. 7,002,621, Reexamination Control 90/014,457

## I. BACKGROUND OF THE REQUEST

Claims 1-3 (the “Challenged Claims”) of U.S. Patent No. 8,283,522 (the “’522 patent” or “’522”) (Ex. 1001) are invalid for obviousness-type double patenting over the claims of U.S. Patent No. 8,916,752 (the “’752 patent”) (Ex. 1003), which is still active. Both patents are owned by Corteva and name the same inventors: Terry R. Wright, Justin M. Lira, Terence Anthony Walsh, Donald J. Merlo, Gaofeng Lin, Jayakumar P. Samuel. The ‘752 patent is a continuation of the ‘522 patent. The relationships among the patents are shown in the family tree illustrated below:



The ‘522 patent was filed on November 3, 2008 and has been granted 907 days of patent term adjustment (PTA) which results in an expiry date of April 21, 2029. The ‘752 patent was filed on October 8, 2012 and its expiry is October 27, 2026. There has been no terminal disclaimer filed for the ‘522 patent over the ‘752 patent. However, there has been a terminal disclaimer filed for the ‘752 patent over the ‘522 patent that was filed in response to an obviousness-type double patenting rejection in light of the ‘522 patent.

The Challenged Claims are generally directed to a plant cell with a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity. Plants are then composed of a plurality of these cells. Due to enhanced aryloxyalkanoate dioxygenase activity in the plant cells, plants composed of these cells are resistant or tolerant of aryloxyalkanoate-based herbicides. As a result, a crop field that is composed of a multitude of these plants can be treated with

aryloxyalkanoate-based herbicide to control the growth of weeds while minimizing deleterious effects on desired crops.

The Patent Owner has already obtained and enjoyed patent protection for these genetically altered plant cells in the '752 patent. Because the claimed invention of the '522 patent is not patentably distinct from the claims of the '752 patent, Patent Owner would continue to enjoy an unjustified extension of patent exclusivity beyond the expiration of the '752 patent's term. Furthermore, the '522 does not benefit from a safe harbor because the '522 patent and the '752 patent did not issue from divisional applications of one another. 35 U.S.C. §121. The minor difference in the claim sets are only obvious features of the art, taking claim 1 as an example below:

'522 Patent Claim 1	'752 Patent Claim 1
<p>[1.pre] A plant cell comprising</p> <p>[1.a] a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.</p>	<p>[1.pre] A transgenic plant cell comprising</p> <p>[1.a] a recombinant polynucleotide that encodes a protein having an activity that catalyzes degradation of a herbicidal compound selected from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.</p>

'752 claim 1 recites "a transgenic plant cell comprising a recombinant polynucleotide that catalyzes degradation of a herbicidal compound from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin". The main difference between claim 1 of the '522 patent and claim 1 of the '752 patent is that the polynucleotide encodes a protein with "aryloxyalkanoate

dioxygenase activity”. Notably, phenoxypropionic and phenoxyacetic auxin, claimed in the ‘752, are composed of aryloxyalkanoate structures. Ex. 1003, 3:37-45. The specification of the ‘752 patent explains the relationship between the claimed “aryloxyalkanoate dioxygenase activity” and “activity that catalyzes degradation of a herbicidal compound selected from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin.” Additionally, SEQ ID NO:2 and SEQ ID NO:4 correspond to the same sequences in both specifications for both the ‘522 patent and the ‘752 patent as detailed below. Similar arguments apply to the other Challenged Claims, as discussed in Section III. Accordingly, the Challenged Claims of the ‘522 patent are unpatentable due to obviousness-type double patenting of claims 1, 16, and 18 of the ‘752 patent.

'522 Patent SEQ ID NO: 2	'752 Patent SEQ ID NO: 2
<p>&lt;210&gt; SEQ ID NO: 2</p> <p>&lt;211&gt; LENGTH: 292</p> <p>&lt;212&gt; TYPE: CDS</p> <p>&lt;213&gt; ORGANISM: Delftia acidovorans</p> <p>&lt;400&gt; SEQUENCE: 2</p> <p>Met Gln Thr Thr Leu Gln Ile Thr Pro Thr Gly Ala Thr Leu Gly Ala 1 5 10 15</p> <p>Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala Gly Phe Ala 20 25 30</p> <p>Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro Gly 35 40 45</p> <p>Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe Gly 50 55 60</p> <p>Ala Phe Gln Arg Ile Gly Gly Gly Asp Ile Val Ala Tyr Ser Asn Val 65 70 75 80</p> <p>Lys Ala Asp Gly Thr Val Arg Gln His Ser Ser Ala Gln Trp Asp Asp 85 90 95</p> <p>Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser Thr 100 105 110</p> <p>Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val Val 115 120 125</p> <p>Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala Tyr 130 135 140</p> <p>Asp Ala Leu Asp Gln Ala Thr Arg Ala Leu Val His Gln Arg Ser Ala 145 150 155 160</p> <p>Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln Gln 165 170 175</p> <p>Ala Gly Ser Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr Pro 180 185 190</p> <p>Leu Arg Pro Leu Val Lys Val His Pro Gln Thr Gly Arg Pro Ser Leu 195 200 205</p> <p>Leu Phe Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala Gln 210 215 220</p> <p>Ser Gln Arg Phe Leu Gln Gly Leu Val Asp Trp Ala Cys Gln Ala Pro 225 230 235 240</p> <p>Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp Asp 245 250 255</p> <p>Asn Arg Cys Leu Leu His Arg Ala Gln Pro Trp Asp Phe Lys Leu Pro 260 265 270</p> <p>Arg Val Met Trp His Ser Arg Leu Ala Gly Asp Pro Gln Thr Gln Gly 275 280 285</p> <p>Ala Ala Leu Val 290</p>	<p>&lt;210&gt; SEQ ID NO: 2</p> <p>&lt;211&gt; LENGTH: 292</p> <p>&lt;212&gt; TYPE: CDS</p> <p>&lt;213&gt; ORGANISM: Delftia acidovorans</p> <p>&lt;400&gt; SEQUENCE: 2</p> <p>Met Gln Thr Thr Leu Gln Ile Thr Pro Thr Gly Ala Thr Leu Gly Ala 1 5 10 15</p> <p>Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala Gly Phe Ala 20 25 30</p> <p>Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro Gly 35 40 45</p> <p>Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe Gly 50 55 60</p> <p>Ala Phe Gln Arg Ile Gly Gly Gly Asp Ile Val Ala Tyr Ser Asn Val 65 70 75 80</p> <p>Lys Ala Asp Gly Thr Val Arg Gln His Ser Ser Ala Gln Trp Asp Asp 85 90 95</p> <p>Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser Thr 100 105 110</p> <p>Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val Val 115 120 125</p> <p>Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala Tyr 130 135 140</p> <p>Asp Ala Leu Asp Gln Ala Thr Arg Ala Leu Val His Gln Arg Ser Ala 145 150 155 160</p> <p>Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln Gln 165 170 175</p> <p>Ala Gly Ser Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr Pro 180 185 190</p> <p>Leu Arg Pro Leu Val Lys Val His Pro Gln Thr Gly Arg Pro Ser Leu 195 200 205</p> <p>Leu Phe Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala Gln 210 215 220</p> <p>Ser Gln Arg Phe Leu Gln Gly Leu Val Asp Trp Ala Cys Gln Ala Pro 225 230 235 240</p> <p>Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp Asp 245 250 255</p> <p>Asn Arg Cys Leu Leu His Arg Ala Gln Pro Trp Asp Phe Lys Leu Pro 260 265 270</p> <p>Arg Val Met Trp His Ser Arg Leu Ala Gly Asp Pro Gln Thr Gln Gly 275 280 285</p> <p>Ala Ala Leu Val 290</p>

'522 Patent SEQ ID NO: 4	'752 Patent SEQ ID NO: 4
<p>&lt;210&gt; SEQ ID NO: 4 &lt;211&gt; LENGTH: 295 &lt;212&gt; TYPE: PRT &lt;213&gt; ORGANISM: <i>Dactyloctenium aegyptium</i></p> <p>&lt;400&gt; SEQUENCE: 4</p> <p>Met Ala Gln Thr Thr Leu Gln Ile Thr Pro Thr Gly Ala Thr Leu Gly 1 5 10 15</p> <p>Ala Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala Gly Thr 20 25 30</p> <p>Ala Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Thr Phe Pro 35 40 45</p> <p>Gly Gln His Leu Ser Ser Asp Gln Gln Thr Thr Phe Ala Lys Asp Thr 50 55 60</p> <p>Gly Ala Ile Gln Asp Ile Gly Gly Gly Asp Thr Val Ala Thr Ser Ser 65 70 75 80</p> <p>Val Lys Ala Asp Gly Thr Val Arg Gln His Ser Phe Ala Gln Phe Asp 85 90 95</p> <p>Asp Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser 100 105 110</p> <p>Thr Tyr Met Thr Val Met Ala Gln Gly Ala Val Phe Ser Ala Gln Val 115 120 125</p> <p>Val Pro Ala Val Gly Gly Asp Thr Tyr Phe Ala Asp Met Arg Ala Ala 130 135 140</p> <p>Tyr Asp Ala Leu Asp Gln Ala Thr Asp Ala Leu Val His Gln Asp Ser 145 150 155 160</p> <p>Ala Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln 165 170 175</p> <p>Gln Ala Gly Ser Ala Tyr Phe Gly Tyr Gly Met Asp Thr Thr Ala Thr 180 185 190</p> <p>Phe Leu Asp Phe Leu Val Lys Val His Phe Gln Thr Gly Arg Phe Ser 195 200 205</p> <p>Leu Leu Ile Gly Asp His Ala His Ala Thr Phe Gly Met Asp Ala Ala 210 215 220</p> <p>Gln Ser Gln Asp Phe Leu Gln Gly Leu Val Asp Tyr Ala Gln Gln Ala 225 230 235 240</p> <p>Pro Asp Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Tyr 245 250 255</p> <p>Asp Ser Asp Tyr Leu Leu His Arg Ala Gln Pro Tyr Asp Phe Lys Leu 260 265 270</p> <p>Pro Asp Val Met Tyr His Ser Asp Leu Ala Gly Arg Pro Gln Thr Gln 275 280 285</p> <p>Gly Ala Ala Leu Val 290</p>	<p>&lt;210&gt; SEQ ID NO: 4 &lt;211&gt; LENGTH: 295 &lt;212&gt; TYPE: PRT &lt;213&gt; ORGANISM: <i>Dactyloctenium aegyptium</i></p> <p>&lt;400&gt; SEQUENCE: 4</p> <p>Met Ala Gln Thr Thr Leu Gln Ile Thr Pro Thr Gly Ala Thr Leu Gly 1 5 10 15</p> <p>Ala Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala Gly Thr 20 25 30</p> <p>Ala Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Thr Phe Pro 35 40 45</p> <p>Gly Gln His Leu Ser Ser Asp Gln Gln Thr Thr Phe Ala Lys Asp Thr 50 55 60</p> <p>Gly Ala Ile Gln Arg Ile Gly Gly Gly Asp Thr Val Ala Thr Ser Ser 65 70 75 80</p> <p>Val Lys Ala Asp Gly Thr Val Asp Gln His Ser Thr Ala Gln Phe Asp 85 90 95</p> <p>Asp Met Met Lys Val Thr Val Gly Asn Met Ala Trp His Ala Asp Ser 100 105 110</p> <p>Thr Tyr Met Thr Val Met Ala Gln Gly Ala Val Phe Ser Ala Gln Val 115 120 125</p> <p>Val Phe Ala Val Gly Gly Arg Thr Tyr Phe Ala Asp Met Arg Ala Ala 130 135 140</p> <p>Tyr Asp Ala Leu Asp Gln Ala Thr Arg Ala Leu Val His Gln Asp Ser 145 150 155 160</p> <p>Ala Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln 165 170 175</p> <p>Gln Ala Gly Ser Ala Tyr Phe Gly Tyr Gly Met Asp Thr Thr Ala Thr 180 185 190</p> <p>Phe Leu Asp Phe Leu Val Lys Val His Phe Gln Thr Gly Arg Phe Ser 195 200 205</p> <p>Leu Leu Ile Gly Arg His Ala His Ala Thr Phe Gly Met Asp Ala Ala 210 215 220</p> <p>Gln Ser Gln Asp Phe Leu Gln Gly Leu Val Asp Tyr Ala Gln Gln Ala 225 230 235 240</p> <p>Pro Asp Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Tyr 245 250 255</p> <p>Asp Ser Asp Tyr Leu Leu His Arg Ala Gln Pro Tyr Asp Phe Lys Leu 260 265 270</p> <p>Pro Asp Val Met Tyr His Ser Asp Leu Ala Gly Arg Pro Gln Thr Gln 275 280 285</p> <p>Gly Ala Ala Leu Val 290</p>

## II. SUBSTANTIAL NEW QUESTIONS OF PATENTABILITY

Reexamination is respectfully requested for claims 1-3 (“Challenged Claims”) of the ’522 patent under 35 U.S.C. § 302 and 37 C.F.R. § 1.510.

### A. Listing of Prior Art Patents and Printed Publications

Pursuant to 37 C.F.R. § 1.510(b)(3), reexamination of the Challenged Claims based on obviousness-type double patenting is requested in view of the references below. This Request



sets forth that the Challenged Claims are unpatentable due to obviousness-type double patenting over claims of U.S. Patent No. 8,916,752 to Wright et al. (the “’752 patent” or “’752”).

- Exhibit 1003: U.S. Patent No. 8,916,752 was filed on October 8, 2012 and issued on December 23, 2014 to Wright, Lira, Walsh, Merlo, Lin and Samuel (the “’752 patent” or “’752”) is a double patenting reference for the ’522 patent because it names a common inventor and a common assignee.

A Form SB-08 and copies of the cited references are submitted herewith.

**B. Statement Setting Forth Each Substantial New Question of Patentability**

This request presents a substantial new question of patentability for the ’522 patent based on the doctrine of obviousness-type double patenting in view of the ’752 patent. Obviousness-type double patenting may raise a substantial new question of patentability during reexamination proceedings. *See* MPEP § 804(I)(D) (“A double patenting issue may raise a substantial new question of patentability of a claim of a patent, and thus can be addressed in a reexamination proceeding.”). The issue of obviousness-type double patenting was not previously raised, considered or decided during the original prosecution of the ’522 patent. The ’752 patent is available as an obviousness-type double patenting reference because the ’752 patent shares with the ’522 patent common inventors (Wright, Lira, Walsh, Merlo, Lin and Samuel), common applicant (Dow Agrosiences LLC), and current assignee (Corteva Agriscience LLC).

The ’752 patent would be important to a reasonable examiner considering the patentability of the Challenged Claims of the ’522 patent. During examination of the ’522 patent the issue of obviousness-type double patenting was never raised. The issue of obviousness-type double patenting with respect to the ’522 patent is therefore new. However, during prosecution of the ’752 patent, the Examiner recognized the similarities between the ’522 and ’752 patents

and issued a rejection based on obviousness-type double patenting over the '522 patent. Ex. 1004, 211-212, 266-267.

Accordingly, as detailed in more detail in Section III below, the '752 patent presents a substantial new question of obviousness-type double patenting that has not been raised of addressed by the Patent Office or Patent Owner during prosecution of the '522 patent. Specifically, the Challenged Claims are unpatentable due to obviousness-type double patenting because the challenged claims are not patentably distinct from claims 1, 16 and 18 of the '752 patent. As set forth herein, **claims 1, 2 and 3 of the '522 patent are unpatentable due to obviousness-type double patenting over claims 1, 16 and 18 of the '752 patent** – thus raising a substantial new question of patentability.

### **C. Background and Prosecution of the '522 Patent**

#### **1. The '522 Patent**

The '522 patent describes the ability to generate crops that are resistant to herbicides that contain aryloxyalkanoate chemical substructures. This is an alternative to the use of glyphosate tolerant crops, which are only tolerant to the glyphosate herbicide. The use of the glyphosate herbicide to control weed formation during crop production has resulted in the recent development of glyphosate-resistant weeds.

Generating aryloxyalkanoate-resistant crops allows for the use of many commercialized herbicides, not just glyphosate, to control the formation of weeds, without deleteriously affecting the crops themselves. Although these herbicides contain aryloxyalkanoate structures, they are chemically diverse and the '522 patent asserts that it is unprecedented to introduce tolerance to such a wide scope of herbicides via the introduction of a single gene. In particular, the introduction of this single gene allows for the generation of novel plants that are resistant to both 2,4-D (a common herbicide) along with pyridyloxyacetate herbicides. The patent describes a

preferred enzyme and gene for use in the invention as AAD-12 (AryloxyAlkanoate Dioxygenase).

The '522 patent also discloses the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes. Such enzymes would be capable of degrading phenoxyacetate auxin and/or pyridyloxyacetate auxin herbicides. The AAD-12 gene allows for the production of these enzymes to generate crops that are resistant to a wider scope of auxin herbicides.

## **2. Prosecution History of the '522 Patent**

The '522 issued from U.S. Application No. 12/091,896, which was filed on November 3, 2008. The Examiner issued a Non-Final Office Action on July 14, 2011 rejecting prosecuted claims 89-94 and 103 as being unpatentable over prior art. Ex. 1002 ("'522 File History"), 908-917. An amendment filed on October 14, 2011 limited the claims such that the protein responsible for the enhanced degradation of aryloxyalkanoate herbicides had to have "at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO: 4." Ex. 1002, 926-933. In a Final Office Action issued on March 27, 2012, the Examiner indicated that a majority of the claims were allowable. Ex. 1002, 963-968. The applicant, in an Amendment on May, 29 2012, cancelled the single rejected claim and accepted the allowable subject matter. Ex. 1002, 971-975.

A Notice of Allowance issued on June 11, 2012, though the examiner did not specifically indicate the reasons for allowance. Ex. 1002, 983.

## **III. DETAILED EXPLANATION OF THE PERTINENCE AND MANNER OF APPLYING THE PRIOR ART REFERENCES TO EVERY CLAIM FOR WHICH REEXAMINATION IS REQUESTED**

In accordance with 37 C.F.R. § 1.510(b)(2), Requester provides the following detailed explanation of the pertinence and manner of applying claims of the '752 patent to claims 1-3 of

the '522 patent, for which reexamination is requested based upon obviousness-type double patenting.

As explained in greater detail below, the Challenged Claims of the '522 patent are unpatentable due to obviousness-type double patenting over claims 1, 16 and 18 of the '752 patent.

**A. The Level of Ordinary Skill in the Pertinent Art**

A person of ordinary skill in the art ("POSITA") would have had a minimum of a Ph.D. degree in plant biology, genetics, or a related field. A person having this background would understand genetic modification, particularly as it pertains to transgenic plants; and the methods by which transgenes are designed, inserted, and subsequently assessed for activity against herbicides.

**B. Requirements for Obviousness-Type Double Patenting**

**1. Legal Standards**

Double patenting is "appropriate for consideration" for "ordering reexamination under 35 U.S.C. § 304 and during subsequent examination on the merits." MPEP §§ 2258, 804. *See also In re Lonardo*, 119 F. 3d 960, 965-67 (Fed. Cir. 1997) ("Congress intended to include double patenting over a prior patent as a basis for reexamination."). "While often described as a court-created doctrine, obviousness-type double patenting is grounded in the text of the Patent Act." *AbbVie Inc. v. Mathilda & Terrence Kennedy Institute of Rheumatology Trust*, 764 F.3d 1366, 1372 (Fed. Cir. 2014). 35 U.S.C. § 101 states, "Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter ... may obtain *a* patent therefor." "Thus, § 101 forbids an individual from obtaining more than one patent on the same invention, *i.e.*, double patenting." *AbbVie*, 764 F.3d at 1372 (Fed. Cir. 2014); *see also* MPEP

§ 804 (“The doctrine of double patenting seeks to prevent the unjustified extension of patent exclusivity beyond the term of a patent.”).

Double-patenting, including nonstatutory obviousness-type double patenting, “may exist between an issued patent and an application filed by the same inventive entity, a different inventive entity having a common inventor, a common applicant, and/or a common owner/assignee.” MPEP § 804; *see also In re Hubbell*, 709 F.3d 1140, 1146-47, (Fed. Cir. 2013). “A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s).” MPEP § 804(II)(B) (citing *In re Berg*, 140 F.3d 1428 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 1052 (Fed. Cir. 1993)). The doctrine prohibits a party from extending the duration of the right to exclude by a later expiring patent’s claims that are not “patentably distinct” from claims in an earlier expiring patent. *See, e.g., G.D. Searle*, 790 F.3d at 1351; *Eli Lilly & Co. v. Barr Labs., Inc.*, 251 F.3d 955, 967 (Fed. Cir. 2001). “Looking instead to the earliest expiration date of all the patents an inventor has on his invention and its obvious variants best fits and serves the purpose of the doctrine of double patenting.” *Gilead Sciences, Inc. v. Natco Pharma Limited*, 753 F.3d 1208, 1216 (Fed. Cir. 2014), *cert. denied*, 135 S. Ct. 1530 (2015); *see also AbbVie Inc. v. Mathilda & Terence Kennedy Inst. Of Rheumatology Trust*, 764 F.3d 1366, 1374 (Fed. Cir. 2014) (“In *Gilead*, we held that **a later-issued, but earlier-expiring patent could qualify as a double patenting reference, and thus invalidate an earlier-issued, but later expiring patent.**”).<sup>1</sup>

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<sup>1</sup> *See, e.g., Ex parte Collect LLC*, Appeal 2021-004967, Reexamination Control 90/014,452 (PTAB Dec. 1, 2021) at pp. 23-26 (holding that “double patenting is proper under reexamination

“A nonstatutory double patenting rejection, if not based on an anticipation rationale or an ‘unjustified timewise extension’ rationale, is ‘analogous to [a failure to meet] the nonobviousness requirement of 35 U.S.C. 103’ except that the patent disclosure principally underlying the double patenting rejection is not considered prior art.” MPEP § 804(II)(B)(2) (citing *In re Braithwaite*, 379 F.2d 594 (CCPA 1967)).

“An obviousness-type double patenting analysis, which ‘compares claims in an earlier patent to claims in a later patent or application’ consists of two steps. First, the court ‘construes the claim[s] in the earlier patent and the claim[s] in the later patent and determines the differences.’ Second, the court ‘determines whether those differences render the claims patentably distinct.’” *Sun Pharm. Indus., Ltd. v. Eli Lilly & Co.*, 611 F.3d 1381, 1384–85 (Fed. Cir. 2010) (citations omitted). Whether any differences render later claims patentably distinct, however, “cannot be considered in isolation—the claims must be considered as a whole,” because “[t]his part of the obviousness-type double patenting analysis is analogous to an obviousness analysis under 35 U.S.C. § 103” and “requires asking whether the claimed subject

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and cannot be avoided since the claims are not patentably distinct”) (Ex. 1007); *Ex parte Collect LLC*, Appeal 2021-005305, Reexamination Control 90/014,453 (PTAB Dec. 1, 2021) (holding that the Examiner did not err in applying obviousness-type double patenting to two related patents that claimed the same priority date and had different patent term adjustments, and that “later-expiring claims of the challenged patent were properly rejected for obviousness-type double patenting over the earlier-expiring claims of the reference patent”) (Ex. 1008); *Ex parte Collect LLC*, Appeal 2021-005302, Reexamination Control 90/014,454 (PTAB Dec. 1, 2021) (similar holdings) (Ex. 1009); *Ex parte Collect LLC*, Appeal 2021-005046, Reexamination Control 90/014,455 (PTAB Dec. 1, 2021) (similar holdings) (Ex. 1010); *Ex parte Collect LLC*, Appeal 2021-005258, Reexamination Control 90/014,457 (PTAB Dec. 1, 2021) (similar holdings) (Ex. 1011). *See also* Ex. 1012-1016 (the reexamination requests in each of the reexamination proceedings).



matter ‘as a whole’ would have been obvious to one of skill in the art.” *Eli Lilly & Co. v. Teva Parenteral Medicines, Inc.*, 689 F.3d 1368, 1377 (Fed. Cir. 2012), *quoting Amgen Inc. v. Hoffman-La Roche Ltd.*, 580 F.3d 1340, 1361 (Fed. Cir. 2009).

To determine whether a claimed invention is “patentably distinct,” the Patent Office considers “[t]he reasons why a person of ordinary skill in the art would conclude that the invention defined in the claim at issue would have been an obvious variation of the invention defined in a claim in the patent.” MPEP § 804(II)(B). For example, a claimed apparatus or device is not patentably distinct over an earlier expiring claim directed towards a method of using the apparatus or device. *See e.g., In re Lonardo*, 119 F.3d 960, 968 (Fed. Cir. 1997) (“We do not agree that there is a patentable distinction between the method of using the device and the device itself.”); *Geneva Pharm., Inc. v. GlaxoSmithKline PLC*, 349 F.3d 1373, 1385 (Fed. Cir. 2003) (holding that claims to a compound and a method of using the compound were “not patentably distinct”); *In re Chaplin*, 711 F. App'x 644 (Fed. Cir. 2018) (holding that claims that “sought coverage for the virus itself” were not patentably distinct from earlier “claims directed to methods of amplifying and using” that virus).

“Even though the specification of the applied patent or copending application is not technically considered to be prior art, it may still be used to interpret the applied claims.” MPEP § 804(II)(B)(2); *see also In re Basell Poliolefine Italia S.P.A.*, 547 F.3d 1371, 1378 (Fed. Cir. 2008). The “specification can be used as a dictionary to learn the meaning of a term in the claim.” MPEP § 804(II)(B)(2)(a). To determine if the claims at issue “merely define an obvious variation of what is earlier disclosed and claimed,” portions of the specification which “provide support for the reference claims” may also be used to determine whether what is later claimed is an obvious variation. MPEP § 804(II)(B)(2)(a); *see also In re Vogel*, 422 F.2d 438, 441-42

(CCPA 1970). On this point, review of the specification can be particularly helpful because, as noted by the CCPA (and reaffirmed by the Federal Circuit):

The disclosure, however, sets forth at least one tangible embodiment within the claim, and it is less difficult and more meaningful to judge whether that thing has been modified in an obvious manner. It must be noted that this use of the disclosure is not in contravention of the cases forbidding its use as prior art, nor is it applying the patent as a reference under 35 U.S.C. § 103, since only the disclosure of the invention claimed in the patent may be examined.

*Vogel*, 422 F.2d at 441-42; *see also Basell*, 547 F.3d at 1378-79. It is also well settled that “a reference patent’s disclosures of utility” may also be used “to determine the question of obviousness.” *AbbVie*, 764 F.3d at 1380.

Further, because the question of whether the claims at issue are “patentably distinct implicates the question of obviousness under § 103,” it is also proper to consider what has been disclosed by the prior art. *Otsuka Pharm. Co. v. Sandoz, Inc.*, 678 F.3d 1280, 1297 (Fed. Cir. 2012) (citations omitted). For example, in *In re Ward*, 236 F.2d 428, 431 (CCPA 1956), the court found claims unpatentable due to obviousness-type double patenting in view of the combination of the claimed subject matter of a patent having common ownership and a prior art patent. *Id.* at 431; *see also Hartness Int’l Inc. v. Simplimatic Eng’g Co.*, 819 F.2d 1100, 1108-09 (Fed. Cir. 1987) (affirming lower court’s ruling holding that a patent was invalid for obviousness-type double patenting in view of the same applicants’ earlier filed and granted claims and of the prior art); *In re Ockert*, 245 F.2d 467, 469 (CCPA 1957) (“It is also well settled that, in determining whether the claims of an application are patentably distinct from those of a patent it is proper to consider what is disclosed by the prior art.”); *In re Simmons*, 312 F.2d 821, 825 (CCPA 1963).

Because a double patenting analysis based on “obviousness” is “analogous to [a failure to meet] the nonobviousness requirement of 35 U.S.C. 103,” “the factual inquiries set forth in *Graham v. John Deere Co.*, 383 U.S. 1, 148 USPQ 459 (1966) that are applied for establishing a background for determining obviousness under 35 U.S.C. § 103 should typically be considered when making a nonstatutory double patenting analysis based on “obviousness.” MPEP § 804(II)(B)(2). These factual inquiries include “the level of ordinary skill in the pertinent art” and “objective indicia of nonobviousness.” *Id.*

## **2. The One-Way Obviousness-Type Double Patenting Test Applies**

The “one way” test applies to the double patenting issue presented in this Request. *See* MPEP § 804(II)(B)(2)(b); *In re Basell Poliolefine Italia S.P.A.*, 547 F.3d at 1371, 1376 (Fed. Cir. 2008) (quoting *In re Berg*, 140 F.3d 1428, 1432 (Fed. Cir. 1998)). The rarely-applied “two-way” test arose to avoid rejections for obviousness-type double patenting “when the applicants filed first for a basic invention and later for an improvement, but, through no fault of the applicants, the PTO decided the applications in reverse order of filing, rejecting the basic application although it would have been allowed if the applications had been decided in the order of their filing.” *Basell*, 547 F.3d. at 1375–76; *see also* MPEP § 804(II)(B)(2)(c). The two-way test is applicable only “in the unusual circumstance that the PTO is solely responsible for the delay in causing [a] second-filed application to issue prior to [a] first.” *Id.* at 1376 (quoting *Berg*, 140 F.3d at 1437). The Federal Circuit has declined to apply the two-way test where an applicant “had significant control over the rate of prosecution of the application” and has not limited the analysis to the time period during which both applications were pending. *In re Emert*, 124 F.3d 1458, 1461 (Fed Cir. 1997).

“If the application under examination is the later-filed application, or both applications are filed on the same day, only a one-way determination of distinctness is needed in resolving the

issue of double patenting, *i.e.*, whether the invention claimed in the application would have been anticipated by, or an obvious variation of, the invention claimed in the patent.” MPEP § 804(II)(B)(2)(b). When determining if two applications are filed on the same day for purposes of deciding whether the two-way test applies, the applications’ actual filing dates, rather than their effective filing dates, are used. *See In re Janssen Biotech, Inc.*, 880 F.3d 1315, 1325 (Fed. Cir. 2018) (using actual filing dates of the challenged and reference patents when finding that one-way test applied).

The one-way test applies here because the ’522 patent at issue was both filed before the reference ’752 patent and issued before the reference ’752 patent.

### **3. Claim Construction**

For claim construction purposes, the Challenged Claims and the claims of the reference patent are virtually identical, except for the minor changes discussed below. Thus, any term in the ’752 patent claims should be construed in the same manner as the corresponding limitation in the ’522 Challenged Claims unless otherwise noted.

#### **C. The Challenged Claims 1, 2 and 3 of the ’522 Patent Are Unpatentable Due To Obviousness-Type Double Patenting Over ’752 Claims 1, 16 and 18**

The Challenged Claims of the ’522 patent are unpatentable due to obviousness-type double patenting and are not patentably distinct over at least claims 1, 16 and 18 of the ’752 patent. *See* MPEP § 804 (“[d]ouble patenting may exist between an issued patent and an application filed by the same inventive entity, or by a different inventive entity having a common inventor, and/or by a common assignee/owner”); *Hubbell*, 709 F.3d at 1146. The ’522 patent is commonly owned with the ’752 patent, and also names Terry R. Wright, Justin M. Lira, Terence Anthony Walsh, Donald Merlo, Jayakumar P. Samuel and Gaofeng Lin as common inventors.

#### **1. ’522 Claim 1 Obvious over ’752 Claim 1**

Claim 1 of the '522 patent is not patentably distinct over claim 1 of the '752 patent.

Claim 1 of the '752 patent recites a “transgenic plant cell comprising a recombinant polynucleotide” wherein the polynucleotide “encodes a protein having an activity that catalyzes degradation of a herbicidal compound selected from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin.” The main difference between claim 1 of the '522 patent and claim 1 of the '752 patent is that limitation [1.a] of the '522 patent claims a polynucleotide that encodes a protein with “aryloxyalkanaote dioxygenase activity”. However, as explained below and in the '752 patent, “aryloxyalkanaote dioxygenase activity” is an “activity that catalyzes degradation of a herbicidal compound selected from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin.”

Thus, for that reason and the reasons set forth below, challenged claim 1 of the '522 patent is not patentably distinct over claim 1 of the '752 patent and is therefore unpatentable due to obviousness-type double patenting.

**a. [1.pre]**

As shown below, limitation [1.pre] of '522 patent claim 1 is not patentably distinct from limitation [1.pre] of the '752 patent claim 1 because both limitations are the same, except that limitation [1.pre] of the '752 patent claim 1 requires a “transgenic” plant cell. To the extent the '522 preamble is limiting, the narrower limitation of the '752 patent discloses the broader limitation [1.pre] of the '522 patent. This difference does not render claim 1 of the '522 patent patentably distinct.

'522 Patent Claim 1	'752 Patent Claim 1	Redline of '752 (Original Text) vs. '522 (Modified Text)
[1.pre] A plant cell comprising	[1.pre] A transgenic plant cell comprising	[1.pre] A <del>transgenic</del> plant cell comprising

**b. [1.a]**

As shown in the table below, [1.a] of '522 patent claim 1 removes from '752 claim [1.a] a limitation of the polynucleotide being "recombinant." The narrower limitation of the '752 patent discloses the broader limitation of the '522 patent, and therefore the limitation of the '522 patent claim 1 is not patentably distinct.

As further shown in the table below, [1.a] of '522 patent claim 1 adds to '752 claim [1.a] a limitation of the protein having "aryloxyalkanoate dioxygenase activity," which replaces the description of the protein having "an activity that catalyzes degradation of a herbicidal compound selected from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin." This limitation does not render claim 1 of the '522 patent patentably distinct.

'522 Patent Claim 1	'752 Patent Claim 1	Redline of '752 (Original Text) vs. '522 (Modified Text)
[1.a] a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.	[1.a] a recombinant polynucleotide that encodes a protein having an activity that catalyzes degradation of a herbicidal compound selected from the group consisting of a phenoxypropionic auxin and a phenoxyacetic auxin, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.	[1.a] a <del>recombinant</del> polynucleotide that encodes a protein having <u>aryloxyalkanoate dioxygenase</u> <del>an activity that catalyzes</del> <del>degradation of a herbicidal</del> <del>compound selected from the</del> <del>group consisting of a</del> <del>phenoxypropionic auxin and a</del> <del>phenoxyacetic auxin</del> , wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4.



The specification of the '752 patent in at least column 4, lines 51-59, describes “aryloxyalkanoate activity.” Ex. 1003. Notably these lines explain that the “invention ... relates in part to the identification and use of genes encoding **aryloxyalkanoate dioxygenase enzymes** that are capable of **degrading phenoxyacetate auxin** and/or pyridyloxyacetates auxin **herbicides**” and includes “**degradation of 2,4-dichlorophenoxyacetic acid** and other aryloxyalkanoate auxin herbicides by a recombinantly expressed **AAD-12 [AryloxyAlkanoate Dioxygenase] enzyme**” (emphasis added). Therefore, a POSITA would have recognized, or at least found it obvious, that “a polynucleotide that encodes a protein having **aryloxyalkanoate dioxygenase activity**” (as claimed in the '522 patent) is also “a polynucleotide that encodes a protein having **an activity that catalyzes degradation of a herbicidal compound** selected from the group consisting of a phenoxypropionic auxin and a **phenoxyacetic auxin**” (as claimed in the '752 patent). Therefore this limitation does not render claim 1 of the '522 patent patentably distinct.

## **2. '522 Claim 2 Obvious over '752 Claim 16**

Claim 2 of the '522 patent is not patentably distinct over claim 16 of the '752 patent. Claim 2 of the '522 patent, which is dependent on claim 1 of the '522 patent, is identical to claim 16 of the '752 patent, which is dependent on claim 1 of the '752 patent. Therefore, the claims are not patentably distinct.

Claim 2 is therefore unpatentable due to obviousness-type double patenting.

'522 Patent Claim 2	'752 Patent Claim 16	Redline of '752 (Original Text) vs. '522 (Modified Text)
[2.pre] A plant comprising	[16.pre] A plant comprising	A plant comprising
[2.a] a plurality of cells of claim 1.	[16.a] a plurality of cells of claim 1.	a plurality of cells of claim 1.

### 3. '522 Claim 3 Obvious over '752 Claim 18

Claim 3 of the '522 patent is not patentably distinct over claim 18 of the '752 patent.

Claim 3 of the '522 patent, which is dependent on claim 1 of the '522 patent, and claim 18 of the '752 patent, which is dependent on claim 1 of the '752 patent, involve a method of controlling weeds in a field with plants having cells with the polynucleotide from claim 1, which would render the plants resistant to aryloxyalkanoate herbicides. As detailed below, claim 3 of the '522 patent includes trivial modifications and additions that do not render claim 3 of the '522 patent patentably distinct from claim 18 of the '752 patent.

Claim 3 of '522 patent reorganizes, and makes trivial modifications to, many limitations of claim 18 of the '752 patent. For example, claim 18 of the '752 patent recites “weeds in a field,” the “field containing a crop,” and the “crop comprising plant cells of claim 1,” whereas claim 3 of the '522 patent recites “weeds in a crop field,” the “crop field comprising a plurality of plants,” and each “plant comprising a plurality of plant cells of claim 1.” The different arrangements of these terms are not patentably distinct, as they each recite plant cells of claim 1 in plants in a crop field with weeds, and both apply an aryloxyalkanoate herbicide to the crop field.

Claim 3 also adds a limitation “wherein expression of said polynucleotide renders said plant resistant or tolerant to “said aryloxyalkanoate herbicide”. The specification of the '752 patent describes “Vectors comprising an AAD-12 polynucleotide” (Ex. 1003, 23:17-18), further noting that “[s]everal techniques exist for introducing foreign recombinant vectors into plant cells, and

for obtaining plants that stably maintain and express the introduced gene” (Ex. 1003, 24:52-54), where “AAD-12” stands for “AryloxyAlkanoate Dioxygenase” which further is detailed in the specification (Ex. 1003, 4:36-37). Further, the ’752 patent describes the invention in terms of “degradation of ... aryloxyalkanoate auxin herbicides by a recombinantly expressed AAD-12 [AryloxyAlkanoate Dioxygenase] enzyme.” Ex. 1003, 4:56-58. Therefore, the additional “wherein” clause of claim 3 of the ’522 patent is merely reciting a property or intended use of the claimed “plant cells of claim 1” in “controlling weeds in a field” with “an aryloxyalkanoate herbicide,” and it would, at the least, be obvious to a POSITA that a polynucleotide encoding “AryloxyAlkanoate Dioxygenase” would lead to resistance against an “aryloxyalkanoate herbicide.” The added limitation does not render claim 3 of the ’522 patent patentably distinct from claim 18 of the ’752 patent.

Claim 3 is therefore unpatentable due to obviousness-type double patenting.

'522 Patent Claim 3	'752 Patent Claim 18	Redline of '752 (Original Text) vs. '522 (Modified Text)
A method of controlling weeds in a crop field, said method comprising applying an aryloxyalkanoate herbicide to said crop field, said crop field comprising a plurality of plants, each said plant comprising a plurality of plant cells of claim 1, wherein expression of said polynucleotide renders said plant resistant or tolerant to said aryloxyalkanoate herbicide.	A method for selectively controlling weeds in a field, said field containing a crop, said crop comprising plant cells of claim 1, said method comprising applying an aryloxyalkanoate herbicide to at least a portion of the crop and the weeds in the field to control the weeds.	A method <del>for selectively of</del> controlling weeds in a <u>crop field</u> , <del>said field containing a crop, said crop comprising plant cells of claim 1;</del> said method comprising applying an aryloxyalkanoate herbicide to <u>said at least a portion of the crop field and the weeds in the field to control the weeds, said crop field comprising a plurality of plants, each said plant comprising a plurality of plant cells of claim 1,</u> wherein expression of said polynucleotide renders said plant resistant or tolerant to said aryloxyalkanoate herbicide.

#### IV. SECONDARY CONSIDERATIONS

Secondary considerations are not considered in an obviousness-type double patenting analysis. *See Procter & Gamble Co. v. Teva Pharm. USA, Inc.*, 566 F.3d 989, 999 (Fed. Cir. 2009) (“double patenting does not require inquiry into objective criteria suggesting non-obviousness”) (citing *Geneva Pharm., Inc. v. GlaxoSmithKline PLC*, 349 F.3d 1373, 1377 n.1 (Fed. Cir. 2003)).

**V. CONCLUSION**

The Commissioner is hereby authorized to charge Deposit Account 18-1945 under Order No. 118525-0001-001 the *Ex Parte* Reexamination fee of \$6,300 under 37 C.F.R. § 1.20(c)(1). Requester believes no other fee is due with this submission, however the Commissioner is hereby authorized to charge any fee deficiency or credit any over-payment to Deposit Account 18-1945.

Please direct all correspondence in this matter to the undersigned.

Dated: November 18, 2022

Respectfully submitted,

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# **Exhibit P**



US010167483B2

(12) **United States Patent**  
**Wright et al.**(10) **Patent No.:** **US 10,167,483 B2**(45) **Date of Patent:** **\*Jan. 1, 2019**(54) **HERBICIDE RESISTANCE GENES**(71) Applicant: **Dow AgroSciences LLC**, Indianapolis, IN (US)(72) Inventors: **Terry R. Wright**, Carmel, IN (US);  
**Justin M. Lira**, Zionsville, IN (US);  
**Terence Anthony Walsh**, Zionsville, IN (US); **Donald Merlo**, Carmel, IN (US);  
**Jayakumar Pon Samuel**, Carmel, IN (US); **Gaofeng Lin**, Zionsville, IN (US)(73) Assignee: **Dow AgroSciences LLC**, Indianapolis, IN (US)

(\*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 527 days.

This patent is subject to a terminal disclaimer.

(21) Appl. No.: **14/491,197**(22) Filed: **Sep. 19, 2014**(65) **Prior Publication Data**US 2015/0080218 A1 Mar. 19, 2015  
US 2017/0009214 A9 Jan. 12, 2017**Related U.S. Application Data**

(63) Continuation of application No. 13/647,081, filed on Oct. 8, 2012, now Pat. No. 8,916,752, which is a continuation of application No. 12/091,896, filed as application No. PCT/US2006/042133 on Oct. 27, 2006, now Pat. No. 8,283,522.

(60) Provisional application No. 60/731,044, filed on Oct. 28, 2005.

(51) **Int. Cl.**  
**C12N 15/82** (2006.01)  
**C12N 9/02** (2006.01)(52) **U.S. Cl.**  
CPC ..... **C12N 15/8274** (2013.01); **C12N 9/0069** (2013.01); **C12N 9/0071** (2013.01); **C12N 15/8275** (2013.01); **C12Y 113/11** (2013.01)(58) **Field of Classification Search**  
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See application file for complete search history.(56) **References Cited****U.S. PATENT DOCUMENTS**4,683,195 A 7/1987 Mullis et al.  
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(Continued)

*Primary Examiner* — David H Kruse(74) *Attorney, Agent, or Firm* — Barnes & Thornburg LLP(57) **ABSTRACT**

The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The preferred enzyme and gene for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide tolerant crop trait and selectable marker opportunities.

**25 Claims, 3 Drawing Sheets****Specification includes a Sequence Listing.**

## US 10,167,483 B2

Page 2

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U.S. Patent

Jan. 1, 2019

Sheet 1 of 3

US 10,167,483 B2

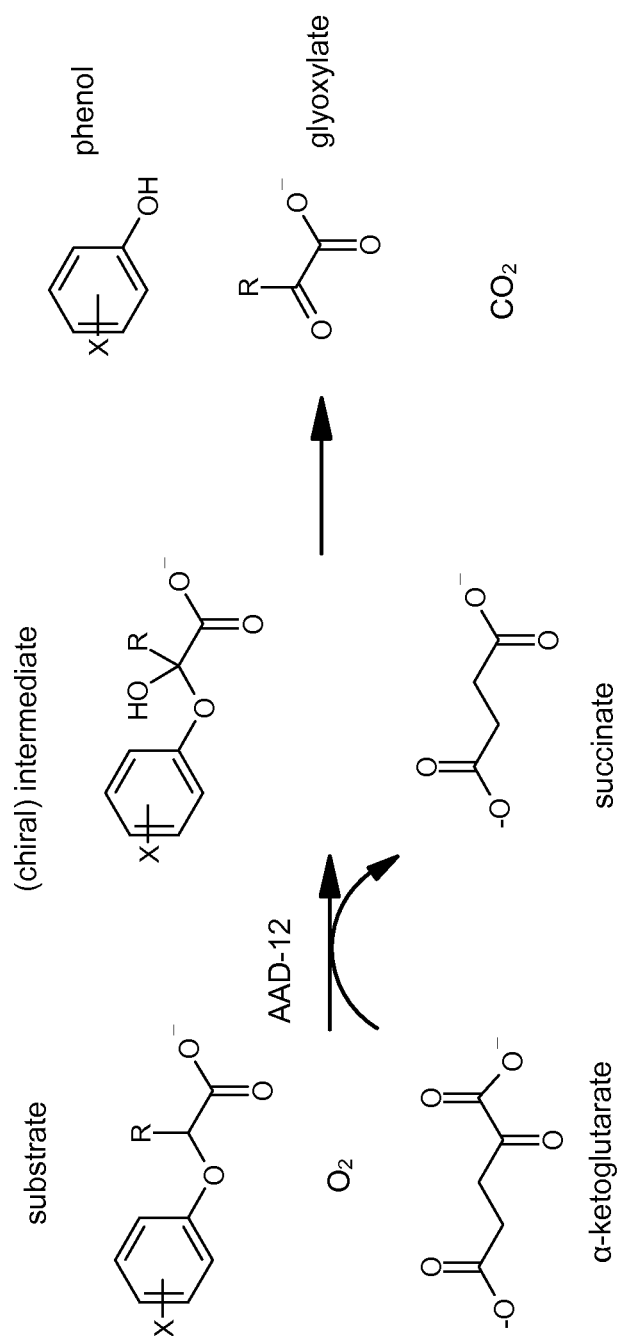


FIG. 1

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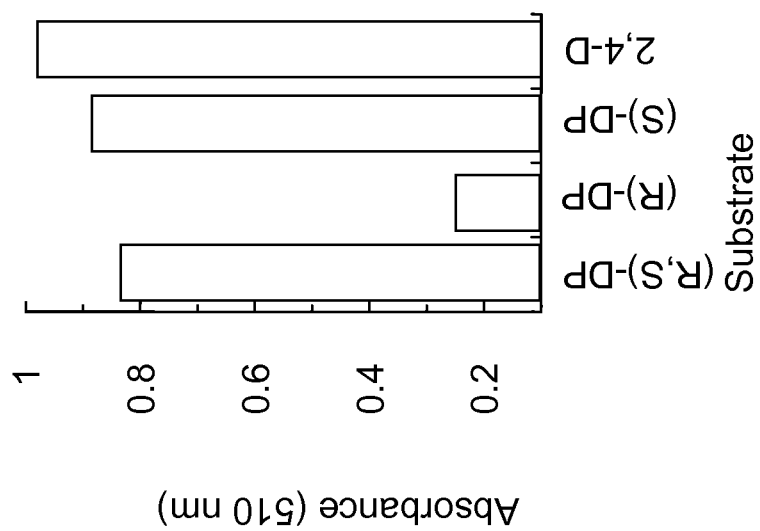
**Jan. 1, 2019**

Sheet 2 of 3

US 10,167,483 B2

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FIG. 2

**U.S. Patent****Jan. 1, 2019****Sheet 3 of 3****US 10,167,483 B2****FIG. 3**

US 10,167,483 B2

1

## HERBICIDE RESISTANCE GENES

## CROSS REFERENCES

This application is a continuation of U.S. patent application Ser. No. 13/647,081, filed on Oct. 8, 2012, which is a continuation of U.S. Pat. No. 8,283,522 with Ser. No. 12/091,896, filed on Nov. 3, 2008, which claims the benefit of PCT International Application Serial No. PCT/US2006/042133, filed on Oct. 27, 2006, which claims the benefit of U.S. Provisional Application Ser. No. 60/731,044, filed on Oct. 28, 2005, the disclosures each of which are expressly incorporated herein by reference.

## BACKGROUND OF THE INVENTION

Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant soybeans, for example, can be sprayed to control weeds without significantly damaging the soybean plants.

With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture.

Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. Alfalfa was the first perennial GTC introduced, furthering the opportunity for repeated use of glyphosate on the same crop and fields repeatedly over a period of years. More GTCs (e.g., wheat, rice, sugar beets, turf, etc.) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (e.g., sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, *petunia*, and begonias; see "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target species (i.e., weed shifts). (Powles and Preston, 2006, Ng et al., 2003; Simarmata et al., 2003; Lorraine-Colwill et al.,

2

2003; Sfiligoj, 2004; Miller et al., 2003; Heap, 2005; Murphy et al., 2002; Martin et al., 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past five years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum*, *Lolium multiflorum*, *Eleusine indica*, *Sorghum halepense*, *Ambrosia artemisiifolia*, *Conyza canadensis*, *Conyza bonariensis*, *Plantago lanceolata*, *Amaranthus palmerii*, and *Amaranthus rudis*. Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea*, *Amaranthus*, *Chenopodium*, *Taraxacum*, and *Commelina* species.

In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burn-down treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agrilience, 2005).

2,4-D is in the phenoxy acid class of herbicides, as is MCPA. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known. Triclopyr and fluroxypyr are pyridyloxyacetic acid herbicides whose mode of action is as a synthetic auxin, also.

These herbicides have different levels of selectivity on certain plants (e.g., dicots are more sensitive than grasses). Differential metabolism by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete mineralization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon



US 10,167,483 B2

3

source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D currently formulated has a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an  $\alpha$ -ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al. (1989), Lyon et al. (1989), Lyon (1993), and U.S. Pat. No. 5,608,147).

A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with  $\alpha$ -ketoglutarate dioxygenase  $\text{Fe}^{+2}$  dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

One unique example with low homology to *tfdA* (31% amino acid identity) is *sdpA* from *Delfia acidovorans* (Kohler et al., 1999, Westendorf et al., 2002, Westendorf et al., 2003). This enzyme has been shown to catalyze the first step in (S)-dichlorprop (and other (S)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf et al., 2003). Transformation of this gene into plants, has not heretofore been reported.

Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxyacetate auxins (such as 2,4-D and dichlorprop), pyridyloxyacetate auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. A multifunctional enzyme for the degradation of herbicides covering multiple modes of action has recently been described (PCT US/2005/014737; filed May 2, 2005). Another unique multifunctional enzyme and potential uses are described hereafter.

#### BRIEF SUMMARY OF THE INVENTION

The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbi-

4

cides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, ALS- (imidazolinone, sulfonylurea), aryloxyalkanoate-, HPPD-, PPO-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, MCPA, triclopyr, fluroxypyr, and one or more commercially available herbicides (e.g., glyphosate, glufosinate, paraquat, ALS-inhibitors (e.g., sulfonylureas, imidazolinones, triazolopyrimidine sulfonanilides, et al), HPPD inhibitors (e.g. mesotrione, isoxaflutole, et al.), dicamba, bromoxynil, aryloxyphenoxypropionates, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including pre-plant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and pyridyloxyacetate herbicides. No  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of both the phenoxyacetate and pyridyloxyacetates auxin herbicides. The preferred enzyme and gene for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide-tolerant crop (HTC) trait and selectable marker opportunities. Plants of the subject invention can be resistant throughout their entire life cycle.

There was no prior motivation to produce plants comprising an AAD-12 gene (preferably an AAD-12 polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an AAD-12 enzyme to render the plants resistant a phenoxyacetic acid herbicide (such as 2,4-D) and/or one or more pyridyloxyacetates herbicides such as triclopyr and fluroxy-

US 10,167,483 B2

5

pyr. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxyacetate auxin and/or pyridyloxyacetates auxin herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid and other aryloxyalkanoate auxin herbicides by a recombinantly expressed AAD-12 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more pyridyloxyacetate or phenoxyacetate auxin herbicides to plants comprising an AAD-12 gene. The subject invention also provides methods of using an AAD-12 gene as a selectable marker for identifying plant cells and whole plants transformed with AAD-12, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates the general chemical reaction that is catalyzed by AAD-12 enzymes of the subject invention.

FIG. 2 is an amino acid sequence alignment of an exemplified AAD-12 protein (SEQ ID NO:2), TfdA (SEQ ID NO:18), AAD-2 (SEQ ID NO:19), AAD-1 (SEQ ID NO 20), and TauD (SEQ ID NO:21).

FIG. 3 illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop.

#### BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the nucleotide sequence of AAD-12 from *Delftia acidovorans*.

SEQ ID NO:2 is the translated protein sequence encoded by SEQ ID NO: 1.

SEQ ID NO:3 is the plant optimized nucleotide sequence of AAD-12 (v1).

SEQ ID NO:4 is the translated protein sequence encoded by SEQ ID NO:3.

SEQ ID NO:5 is the *E. coli* optimized nucleotide sequence of AAD-12 (v2).

SEQ ID NO:6 is the sequence of the M13 forward primer.

SEQ ID NO:7 is the sequence of the M13 reverse primer.

SEQ ID NO:8 is the sequence of the forward AAD-12 (v1) PTU primer.

SEQ ID NO:9 is the sequence of the reverse AAD-12 (v1) PTU primer.

SEQ ID NO:10 is the sequence of the forward AAD-12 (v1) coding PCR primer.

SEQ ID NO:11 is the sequence of the reverse AAD-12 (v1) coding PCR primer.

SEQ ID NO:12 shows the sequence of the “sdpacodF” AAD-12 (v1) primer.

6

SEQ ID NO:13 shows the sequence of the “sdpacodR” AAD-12 (v1) primer.

SEQ ID NO:14 shows the sequence of the “NcoI of Brady” primer.

SEQ ID NO:15 shows the sequence of the “SacI of Brady” primer.

SEQ ID NO:16 provides the sequence of forward primer brjap 5' (speI).

SEQ ID NO:17 provides the sequence of reverse primer brjap 3' (xhoI).

SEQ ID NO:18 provides the sequence of tfdA.

SEQ ID NO:19 provides the sequence of AAD-2.

SEQ ID NO:20 provides the sequence of AAD-1.

SEQ ID NO:21 provides the sequence of tauD.

#### DETAILED DESCRIPTION OF THE INVENTION

The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of the subject herbicide tolerance trait for 2,4-D is its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism, and the like. An additional benefit of the AAD-12 gene is that unlike all tfdA homologues characterized to date, AAD-12 is able to degrade the pyridyloxyacetates auxins (e.g., triclopyr, fluroxypyr) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid). See Table 1. A general illustration of the chemical reactions catalyzed by the subject AAD-12 enzyme is shown in FIG. 1. (Addition of O<sub>2</sub> is stereospecific; breakdown of intermediate to phenol and glyoxylate is spontaneous.) It should be understood that the chemical structures in FIG. 1 illustrate the molecular backbones and that various R groups and the like (such as those shown in Table 1) are included but are not necessarily specifically illustrated in FIG. 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the AAD-12 gene in plants affords protection to a much wider spectrum of auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled. The subject invention can also be used to protect from drift or other off-site synthetic auxin herbicide injury for the full breadth of commercially available phenoxy auxins. Table 1 defines commercially available pyridyloxy and phenoxy auxins and provides relevant chemical structures.

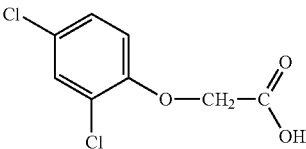
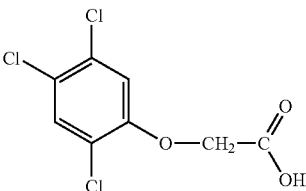
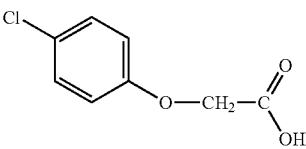
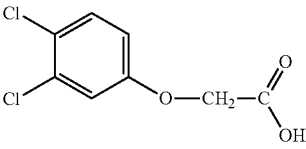
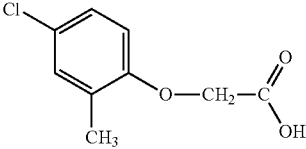
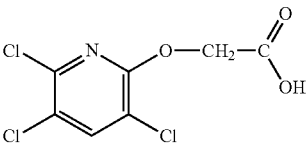
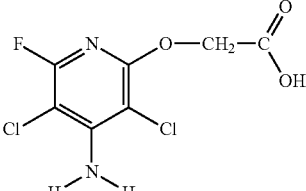
US 10,167,483 B2

7

8

TABLE 1

Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme in planta as general plant esterases convert these esters to the active acids in planta. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Chemical name	CAS no	Possible use rate ranges (g ae/ha)	Preferred use rate ranges (g ae/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	
2,4,5-T	93-76-5	25-4000	25-4000	
4-CPA	122-88-3	25-4000	25-4000	
3,4-DA	588-22-7	25-4000	25-4000	
MCPA	94-74-6	25-4000	125-1550	
Triclopyr	55335-06-3	50-2000	70-840	
Fluroxypyr	69377-81-7	25-2000	35-560	

A single gene (AAD-12) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Addition-

ally, AAD-12 can provide protection in planta to pyridyloxyacetate herbicides where natural tolerance also was not sufficient to allow selectivity, expanding the potential utility of these herbicides. Plants containing AAD-12 alone now may be treated sequentially or tank mixed with one, two, or

US 10,167,483 B2

9

a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several pyridyloxyacetate auxin compounds may be applied to plants expressing AAD-12 with reduced risk of injury from said herbicides. The rate for each pyridyloxyacetate herbicide may range from 25 to 2000 g ae/ha, and more typically from 35-840 g ae/ha for the control of additional dicot weeds.

Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking AAD-12 with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant dicot weed species in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (e.g., 2,4-D) and pyridyloxyacetates auxin herbicides (e.g., triclopyr)-selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from approximately 2 hours to approximately 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within about 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-12 stack could range from about 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from about 25-4000 g ae/ha; and pyridyloxyacetates auxin herbicide(s) (one or more) could be applied from 25-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

Plantlets are typically resistant throughout the entire growing cycle. Transformed plants will typically be resistant to new herbicide application at any time the gene is expressed. Tolerance is shown herein to 2,4-D across the life cycle using the constitutive promoters tested thus far (primarily CsVMV and AtUbi 10). One would typically expect this, but it is an improvement upon other non-metabolic activities where tolerance can be significantly impacted by the reduced expression of a site of action mechanism of resistance, for example. One example is Roundup Ready cotton, where the plants were tolerant if sprayed early, but if sprayed too late the glyphosate concentrated in the meristems (because it is not metabolized and is translocated); viral promoters Monsanto used are not well expressed in the flowers. The subject invention provides an improvement in these regards.

10

Herbicide formulations (e.g., ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (e.g., adjuvants, surfactants, drift retardants, or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant or resistant weed species. This could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (e.g., resistant plant or bacterial EPSPS, glyphosate oxidoreductase (GOX), GAT), glufosinate resistance (e.g., Pat, bar), acetolactate synthase (ALS)-inhibiting herbicide resistance (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrimidinylthiobenzoates, and other chemistries=AHAS, Csrl, SurA, et al.), bromoxynil resistance (e.g., Bxn), resistance to inhibitors of HPPD (4-hydroxyphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (e.g., psbA), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (e.g., PPO-1), resistance to phenylurea herbicides (e.g., CYP76B1), dicamba-degrading enzymes (see, e.g., US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes. In vivo modified EPSPS can be used in some preferred embodiments, as well as Class I, Class II, and Class III glyphosate resistance genes.

Regarding additional herbicides, some additional preferred ALS inhibitors include but are not limited to the sulfonylureas (such as chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron), imidazolinones (such as imazamox, imazethapyr, imazaquin), triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyriithobac), and flucarbazone. Some preferred HPPD inhibitors include but are not limited to mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include but are not limited to flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

Additionally, AAD-12 alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to tfdA, the genes of the subject invention can still be generally classified in the same overall



US 10,167,483 B2

11

family of  $\alpha$ -ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "HX(D/E)X<sub>23-26</sub>(T/S)X<sub>114-183</sub>HX<sub>10-13</sub>R" motif which comprises the active site. The histidines coordinate Fe<sup>+2</sup> ion in the active site that is essential for catalytic activity (Hogan et al., 2000). The preliminary in vitro expression experiments discussed herein were tailored to help select for novel attributes. These experiments also indicate the AAD-12 enzyme is unique from another disparate enzyme of the same class, disclosed in a previously filed patent application (PCT US/2005/014737; filed May 2, 2005). The AAD-1 enzyme of that application shares only about 25% sequence identity with the subject AAD-12 protein.

More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also pyridyloxyacetate herbicides. No  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase) genes and proteins.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and pyridyloxyacetate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic acids, and pyridyloxyacetic acid herbicides by a recombinantly expressed AAD-12 enzyme.

The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP in vitro. An additional advantage that AAD-12 transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

The subject invention also includes methods of controlling weeds wherein said methods comprise applying a pyridyloxyacetate and/or a phenoxy auxin herbicide to plants comprising an AAD-12 gene.

In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also pyridyloxyacetate herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzyme in the degradation pathway is *tfdA*. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an  $\alpha$ -ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001).

12

*TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al., 1989; Lyon et al., 1989; Lyon et al., 1993). A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, a small collection of  $\alpha$ -ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to *tfdA*.

The subject invention relates in part to surprising discoveries of new uses for and functions of a distantly related enzyme, *sdpA*, from *Delfia acidivorans* (Westendorf et al., 2002, 2003) with low homology to *tfdA* (31% amino acid identity). This  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and S-dichloroprop (Westendorf et al., 2002 and 2003). However, no  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of pyridyloxyacetate chemical class. *SdpA* has never been expressed in plants, nor was there any motivation to do so in part because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-12 proteins and genes. AAD-12 was presently confirmed to degrade a variety of phenoxyacetate auxin herbicides in vitro. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance include the pyridyloxyacetate auxin herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxyacetate and pyridyloxyacetate auxins).

Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic auxin herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-12) capable of degrading phenoxy and/or pyridyloxy auxin herbicides.

The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf weeds. AAD-12 can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits [e.g., glyphosate resistance, glufosinate resistance, ALS-inhibitor (e.g., imidazolinone, sulfonylurea, triazopyrimidine sulfonamide) resistance, bromoxynil resistance, HPPD-inhibitor resistance, PPO-inhibitor resistance, et al.], and insect resistance traits (Cry1F, Cry1Ab, Cry 34/45, other Bt. Proteins, or insecticidal proteins of a non-*Bacillus* origin, et al.) for example. Additionally, AAD-12 can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with AAD-12-containing constructs and have demonstrated high levels of resistance to both the

US 10,167,483 B2

13

phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to “plant optimized” genes that encode proteins of the subject invention.

Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by “acid trapping,” a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTCs. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-12. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (e.g., glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

The transgenic expression of the subject AAD-12 genes is exemplified in, for example, *Arabidopsis*, tobacco, soybean, cotton, rice, corn and canola. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be utilized in multiple other monocot (such as pasture grasses or turf grass) and dicot crops like alfalfa, clover, tree species, et al. Likewise, 2,4-D (or other AAD-12-substrates) can be more positively utilized in grass crops where tolerance is moderate, and increased tolerance via this trait would provide growers the opportunity to use these herbicides at more efficacious rates and over a wider application timing without the risk of crop injury.

Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed. This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-12 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-12 for other aryloxyalkanoate auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

Proteins (and Source Isolates) of the Subject Invention.

The present invention provides functional proteins. By “functional activity” (or “active”) it is meant herein that the proteins/enzymes for use according to the subject invention

14

have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce “an effective amount” of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

One cannot easily discuss the term “resistance” and not use the verb “tolerate” or the adjective “tolerant.” The industry has spent innumerable hours debating Herbicide Tolerant Crops (HTC) versus Herbicide Resistant Crops (HRC). HTC is a preferred term in the industry. However, the official Weed Science Society of America definition of resistance is “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis.” As used herein unless otherwise indicated, herbicide “resistance” is heritable and allows a plant to grow and reproduce in the presence of a typical herbicidally effective treatment by a herbicide for a given plant, as suggested by the current edition of *The Herbicide Handbook* as of the filing of the subject disclosure. As is recognized by those skilled in the art, a plant may still be considered “resistant” even though some degree of plant injury from herbicidal exposure is apparent. As used herein, the term “tolerance” is broader than the term “resistance,” and includes “resistance” as defined herein, as well as an improved capacity of a particular plant to withstand the various degrees of herbicidally induced injury that typically result in wild-type plants of the same genotype at the same herbicidal dose.

Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein’s amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybrid-



US 10,167,483 B2

15

ize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbent assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria.

Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutant strains can also be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook et al., 1989, which is incorporated herein by reference.

#### Polynucleotides and Probes.

The subject invention further provides nucleic acid sequences that encode proteins for use according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of inter-

16

est. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the AAD-12 gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage. In addition, desired levels and times of expression can also depend on the type of plant and the level of tolerance desired. Some preferred embodiments use strong constitutive promoters combined with transcription enhancers and the like to increase expression levels and to enhance tolerance to desired levels. Some such applications are discussed in more detail below, before the Examples section.

As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein in vivo, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an

## US 10,167,483 B2

17

appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, etc.).

As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G. H., M. M. Manak (1987) DNA Probes, Stockton Press, New York, N.Y., pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2×SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1×SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1×SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2×SSC/0.1% SDS can be prepared by adding 50 ml of 20×SSC and 5 ml of 10% SDS to 445 ml of water. 20×SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is, one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with <sup>32</sup>P-

18

labeled gene-specific probes can be performed by standard methods (see, e.g., Maniatis et al. 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C. below the melting temperature (T<sub>m</sub>) of the DNA hybrid in 6×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz et al. 1983):

$$T_m = 81.5^\circ \text{C} + 16.6 \log [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.61(\% \text{ formamide}) - 600/\text{length of duplex in base pairs.}$$

Washes can typically be carried out as follows:

- (1) Twice at room temperature for 15 minutes in 1×SSPE, 0.1% SDS (low stringency wash).
- (2) Once at T<sub>m</sub>-20° C. for 15 minutes in 0.2×SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C. below the melting temperature (T<sub>m</sub>) of the hybrid in 6×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T<sub>m</sub> for oligonucleotide probes can be determined by the following formula:

$$T_m(^{\circ} \text{C.}) = 2(\text{number T/A base pairs}) + 4(\text{number G/C base pairs})$$

(Suggs et al., 1981).

Washes can typically be out as follows:

- (1) Twice at room temperature for 15 minutes 1×SSPE, 0.1% SDS (low stringency wash).
- (2) Once at the hybridization temperature for 15 minutes in 1×SSPE, 0.1% SDS (moderate stringency wash).

In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

- Low: 1 or 2×SSPE, room temperature
- Low: 1 or 2×SSPE, 42° C.
- Moderate: 0.2× or 1×SSPE, 65° C.
- High: 0.1×SSPE, 65° C.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

PCR Technology.

Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see Mullis, U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki et al., 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other

US 10,167,483 B2

19

primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Taq polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

#### Modification of Genes and Proteins.

The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein.

The top two results of BLAST searches with the native aad-12 nucleotide sequence show a reasonable level of homology (about 85%) over 120 basepairs of sequence. Hybridization under certain conditions could be expected to include these two sequences. See GENBANK Acc. Nos. DQ406818.1 (89329742; *Rhodoferrax*) and AJ6288601.1 (44903451; *Sphingomonas*). *Rhodoferrax* is very similar to *Delftia* but *Sphingomonas* is an entirely different Class phylogenetically.

The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like. Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

In addition, U.S. Pat. No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activ-

20

ity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

"Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

Specific changes to the "active site" of the enzyme can be made to affect the inherent functionality with respect to activity or stereospecificity (see alignment FIG. 2). Muller et al. (2006). The known tauD crystal structure was used as a model dioxygenase to determine active site residues while bound to its inherent substrate taurine. Elkins et al. (2002) "X-ray crystal structure of *Escherichia coli* taurine/alpha-ketoglutarate dioxygenase complexed to ferrous iron and substrates," *Biochemistry* 41(16):5185-5192. Regarding sequence optimization and designability of enzyme active sites, see Chakrabarti et al., PNAS, (Aug. 23, 2005), 102 (34):12035-12040.

Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, or 293 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full complements) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained



US 10,167,483 B2

21

using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, B.t. proteins can be used in a truncated (core protein) form (see, e.g., Höfte et al. (1989), and Adang et al. (1985)). As used herein, the term "protein" can include functionally active truncations.

In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990. BLAST nucleotide searches are performed with the NBLAST program, score=100, wordlength=12. Gapped BLAST can be used as described in Altschul et al., 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. See NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, Md., U.S.A.), was used employing the default parameters. These were: a Gap open-

22

ing penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. Table 2 provides a listing of examples of amino acids belonging to each class.

TABLE 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

#### Optimization of Sequence for Expression in Plants.

To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species. Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

#### Transgenic Hosts.

The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf, legume forages (e.g., alfalfa and clover), pasture

US 10,167,483 B2

23

grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

Although plants can be preferred, the subject invention also includes production of highly active recombinant AAD-12 in a *Pseudomonas fluorescens* (Pf) host strain, for example. The subject invention includes preferred growth temperatures for maintaining soluble active AAD-12 in this host; a fermentation condition where AAD-12 is produced as more than 40% total cell protein, or at least 10 g/L; a purification process results high recovery of active recombinant AAD-12 from a Pf host; a purification scheme which yields at least 10 g active AAD-12 per kg of cells; a purification scheme which can yield 20 g active AAD-12 per kg of cells; a formulation process that can store and restore AAD-12 activity in solution; and a lyophilization process that can retain AAD-12 activity for long-term storage and shelf life.

#### Insertion of Genes to Form Transgenic Hosts.

One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Pat. No. 5,135,867.

Vectors comprising an AAD-12 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the insertion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analy-

24

sis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley et al. (1986); and An et al. (1985).

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary factors. The resulting hybrid individuals have the corresponding phenotypic properties.

In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

US 10,167,483 B2

25

Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, inter alia. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate (e.g., PAT/bar), glyphosate (EPSPS), ALS-inhibitors (e.g., imidazolinone, sulfonyleurea, triazolo-pyrimidine sulfonanilide, et al.), bromoxynil, HPPD-inhibitor resistance, PPO-inhibitors, ACC-ase inhibitors, and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. No. 4,945,050 to Cornell and U.S. Pat. No. 5,141,131 to Dow-Elanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, see U.S. Pat. No. 5,177,010 to University of Toledo; U.S. Pat. No. 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Pat. Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Pat. No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Pat. No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Pat. Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Pat. Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. See U.S. Pat. Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Other direct DNA delivery transformation technology includes aerosol beam technology. See U.S. Pat. No. 6,809,232. Electroporation technology has also been used to transform plants. See WO 87/06614 to Boyce Thompson Institute; U.S. Pat. Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Pat. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful

26

when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al. (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G41; hygromycin resistance; methotrexate resistance, as well as those genes which encode for resistance or tolerance to glyphosate; phosphinothricin (bialaphos or glufosinate); ALS-inhibiting herbicides (imida-



US 10,167,483 B2

27

zolinones, sulfonyleureas and triazolopyrimidine herbicides), ACC-ase inhibitors (e.g., aryloxypropionates or cyclohexanediones), and others such as bromoxynil, and HPPD-inhibitors (e.g., mesotrione) and the like.

In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising et al., 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the uidA locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the uidA locus of *E. coli* as described by Jefferson et al., (1987) to identify transformed cells.

In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, see U.S. Pat. No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for

28

expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Pat. No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Pat. Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

Means of Further Increasing Tolerance or Resistance Levels.

It is shown herein that plants of the subject invention can be imparted with novel herbicide resistance traits without observable adverse effects on phenotype including yield. Such plants are within the scope of the subject invention. Plants exemplified and suggested herein can withstand 2x, 3x, 4x, and 5x typical application levels, for example, of at least one subject herbicide. Improvements in these tolerance levels are within the scope of this invention. For example, various techniques are known in the art, and can foreseeably be optimized and further developed, for increasing expression of a given gene.

One such method includes increasing the copy number of the subject AAD-12 genes (in expression cassettes and the like). Transformation events can also be selected for those having multiple copies of the genes.

Strong promoters and enhancers can be used to "supercharge" expression. Examples of such promoters include the preferred 35T promoter which uses 35S enhancers. 35S, maize ubiquitin, *Arabidopsis* ubiquitin, A.t. actin, and CSMV promoters are included for such uses. Other strong viral promoters are also preferred. Enhancers include 4 OCS and the 35S double enhancer. Matrix attachment regions (MARs) can also be used to increase transformation efficiencies and transgene expression, for example.

Shuffling (directed evolution) and transcription factors can also be used for embodiments according to the subject invention.

Variant proteins can also be designed that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like. See e.g. U.S. Pat. No. 7,058,515; Larson et al., Protein Sci. 2002 11: 2804-2813, "Thoroughly sampling sequence space: Large-scale protein design of structural ensembles."; Cramer et al., *Nature Biotechnology* 15, 436-438 (1997), "Molecular evolution of an arsenate detoxification pathway by DNA shuffling."; Stemmer, W.P.C. 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution. Proc. Natl. Acad. Sci. USA 91: 10747-10751; Stemmer, W.P.C. 1994. Rapid evolution of a protein in vitro by DNA shuffling. Nature 370: 389-391; Stemmer, W.P.C. 1995. Searching sequence space. Bio/Technology 13: 549-553; Cramer, A., Cwirla, S. and Stemmer, W.P.C. 1996. Construction and evolution of antibody-phage libraries by DNA shuffling. Nature Medicine 2: 100-103; and Cramer, A., Whitehorn, E. A., Tate, E. and Stemmer, W.P.C. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. Nature Biotechnology 14: 315-319.

US 10,167,483 B2

29

The activity of recombinant polynucleotides inserted into plant cells can be dependent upon the influence of endogenous plant DNA adjacent the insert. Thus, another option is taking advantage of events that are known to be excellent locations in a plant genome for insertions. See e.g. WO 2005/103266 A1, relating to cry1F and cry1Ac cotton events; the subject AAD-12 gene can be substituted in those genomic loci in place of the cry1F and/or cry1Ac inserts. Thus, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (USPA 20030232410), relating to the use of zinc fingers for targeted recombination. The use of recombinases (cre-lox and flp-frt for example) is also known in the art.

AAD-12 detoxification is believed to occur in the cytoplasm. Thus, means for further stabilizing this protein and mRNAs (including blocking mRNA degradation) are included in aspects of the subject invention, and art-known techniques can be applied accordingly. The subject proteins can be designed to resist degradation by proteases and the like (protease cleavage sites can be effectively removed by re-engineering the amino acid sequence of the protein). Such embodiments include the use of 5' and 3' stem loop structures like UTRs from osmotin, and per5 (AU-rich untranslated 5' sequences). 5' caps like 7-methyl or 2'-O-methyl groups, e.g., 7-methylguanylic acid residue, can also be used. See, e.g.: Proc. Natl. Acad. Sci. USA Vol. 74, No. 7, pp. 2734-2738 (July 1977 *Importance of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis*). Protein complexes or ligand blocking groups can also be used.

Computational design of 5' or 3' UTR most suitable for AAD-12 (synthetic hairpins) can also be conducted within the scope of the subject invention. Computer modeling in general, as well as gene shuffling and directed evolution, are discussed elsewhere herein. More specifically regarding computer modeling and UTRs, computer modeling techniques for use in predicting/evaluating 5' and 3' UTR derivatives of the present invention include, but are not limited to: MFold version 3.1 available from Genetics Corporation Group, Madison, Wis. (see Zucker et al., Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. In RNA Biochemistry and Biotechnology, 11-43, J. Barciszewski & B. F. C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, Dordrecht, N L, (1999); Zucker et al., *Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure*. *J. Mol. Biol.* 288, 911-940 (1999); Zucker et al., RNA Secondary Structure Prediction. In *Current Protocols in Nucleic Acid Chemistry* S. Beaucage, D. E. Bergstrom, G. D. Glick, and R. A. Jones eds., John Wiley & Sons, New York, 11.2.1-11.2.10, (2000)), COVE (RNA structure analysis using covariance models (stochastic context free grammar methods)) v. 2.4.2 (Eddy & Durbin, *Nucl. Acids Res.* 1994, 22: 2079-2088) which is freely distributed as source code and which can be downloaded by accessing the website [genetics.wustl.edu/eddy/software/](http://genetics.wustl.edu/eddy/software/), and FOLDALIGN, also freely distributed and available for downloading at the website [bioinf.au.dk](http://bioinf.au.dk). FOLDALIGN/ (see *Finding the most significant common sequence and structure motifs in a set of RNA sequences*. J. Gorodkin, L. J. Heyer and G. D. Stormo. *Nucleic Acids Research*, Vol. 25, no. 18 pp 3724-3732, 1997; *Finding Common Sequence and Structure Motifs in a set of RNA Sequences*. J. Gorodkin, L. J. Heyer, and G. D. Stormo. *ISMB* 5; 120-123, 1997).

30

Embodiments of the subject invention can be used in conjunction with naturally evolved or chemically induced mutants (mutants can be selected by screening techniques, then transformed with AAD-12 and possibly other genes). Plants of the subject invention can be combined with ALS resistance and/or evolved glyphosate resistance. Aminopyralid resistance, for example, can also be combined or "stacked" with an AAD-12 gene.

Traditional breeding techniques can also be combined with the subject invention to powerfully combine, introgress, and improve desired traits.

Further improvements also include use with appropriate safeners to further protect plants and/or to add cross resistance to more herbicides. (Safeners typically act to increase plants immune system by activating/expressing cP450. Safeners are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism, without compromising weed control efficacy.)

Herbicide safeners include benoxacor, cloquintocet, cyometrinil, dichlormid, dicyclonon, dietholate, fenclorazole, fenclorim, flurazole, fluxofenim, furilazole, isoxadifen, mefenpyr, mephenate, naphthalic anhydride, and oxabestrinil. Plant activators (a new class of compounds that protect plants by activating their defense mechanisms) can also be used in embodiments of the subject invention. These include acibenzolar and probenazole.

Commercialized safeners can be used for the protection of large-seeded grass crops, such as corn, grain *sorghum*, and wet-sown rice, against preplant-incorporated or preemergence-applied herbicides of the thiocarbamate and chloroacetanilide families. Safeners also have been developed to protect winter cereal crops such as wheat against postemergence applications of aryloxyphenoxypropionate and sulfonylurea herbicides. The use of safeners for the protection of corn and rice against sulfonylurea, imidazolinone, cyclohexanedione, isoxazole, and triketone herbicides is also well-established. A safener-induced enhancement of herbicide detoxification in safened plants is widely accepted as the major mechanism involved in safener action. Safeners induce cofactors such as glutathione and herbicide-detoxifying enzymes such as glutathione S-transferases, cytochrome P450 monooxygenases, and glucosyl transferases. Hatzios K K, Burgos N (2004) "Metabolism-based herbicide resistance: regulation by safeners," *Weed Science*: Vol. 52, No. 3 pp. 454-467.

Use of a cytochrome p450 monooxygenase gene stacked with AAD-12 is one preferred embodiment. There are P450s involved in herbicide metabolism; cP450 can be of mammalian or plant origin, for example. In higher plants, cytochrome P450 monooxygenase (P450) is known to conduct secondary metabolism. It also plays an important role in the oxidative metabolism of xenobiotics in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase). Resistance to some herbicides has been reported as a result of the metabolism by P450 as well as glutathione S-transferase. A number of microsomal P450 species involved in xenobiotic metabolism in mammals have been characterized by molecular cloning. Some of them were reported to metabolize several herbicides efficiently. Thus, transgenic plants with plant or mammalian P450 can show resistance to several herbicides.

One preferred embodiment of the foregoing is the use of cP450 for resistance to acetochlor (acetochlor-based products include Surpass®, Keystone®, Keystone LA, Full-Time® and TopNotch herbicides) and/or trifluralin (such as Trellan®). Such resistance in soybeans and/or corn is included in some preferred embodiments. For additional

US 10,167,483 B2

31

guidance regarding such embodiments, see e.g. Inui et al., "A selectable marker using cytochrome P450 monooxygenases for *Arabidopsis* transformation," *Plant Biotechnology* 22, 281-286 (2005) (relating to a selection system for transformation of *Arabidopsis thaliana* via *Agrobacterium tumefaciens* that uses human cytochrome P450 monooxygenases that metabolize herbicides; herbicide tolerant seedlings were transformed and selected with the herbicides acetochlor, amiprofos-methyl, chlorpropham, chlorsulfuron, norflurazon, and pendimethalin); Siminszky et al., "Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides," *PNAS* Vol. 96, Issue 4, 1750-1755, Feb. 16, 1999; Sheldon et al, *Weed Science*: Vol. 48, No. 3, pp. 291-295, "A cytochrome P450 monooxygenase cDNA (CYP71A10) confers resistance to linuron in transgenic *Nicotiana tabacum*"; and "Phytoremediation of the herbicides atrazine and metolachlor by transgenic rice plants expressing human CYP1A1, CYP2B6, and CYP2C19," *J Agric Food Chem.* 2006 Apr. 19; 54(8):2985-91 (relating to testing a human cytochrome p450 monooxygenase in rice where the rice plants reportedly showed high tolerance to chloroacetamides (acetochlor, alachlor, metoachlor, pretiachlor, and thenylchlor), oxyacetamides (mefenacet), pyridazinones (norflurazon), 2,6-dinitroanilines (trifluralin and pendimethalin), phosphamides (amiprofos-methyl, thiocarbamates (pyributicarb), and ureas (chlortoluron)).

There is also the possibility of altering or using different 2,4-D chemistries to make the subject AAD-12 genes more efficient. Such possible changes include creating better substrates and better leaving groups (higher electronegativity).

Auxin transport inhibitors (e.g. diflufenzopyr) can also be used to increase herbicide activity with 2,4-D.

Unless specifically indicated or implied, the terms "a", "an", and "the" signify "at least one" as used herein.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

#### Example 1—Method for Identifying Genes that Impart Resistance to 2,4-D in Planta

As a way to identify genes which possess herbicide degrading activities in planta, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e.,  $\alpha$ -ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with  $\leq 50\%$  homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation

32

(relative to tfdA from *Ralstonia eutropha*) can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

A single gene (sdpA) was identified from the NCBI database (see the [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov) website; accession #AF516752) as a homologue with only 31% amino acid identity to tfdA. Percent identity was determined by first translating both the sdpA and tfdA DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

#### Example 2—Optimization of Sequence for Expression in Plants and Bacteria

##### 2.1—Background.

To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

Table 3 illustrates how high the G+C content is in maize. For the data in Table 3, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (Accelrys, San Diego, Calif.). Intron sequences were ignored in the calculations.

TABLE 3

Compilation of G + C contents of protein coding regions of maize genes		
Protein Class <sup>a</sup>	Range % G + C	Mean % G + C <sup>b</sup>
Metabolic Enzymes (76)	44.4-75.3	59.0 (+/-8.0)
Structural Proteins (18)	48.6-70.5	63.6 (+/-6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (+/-4.9)



US 10,167,483 B2

33

TABLE 3-continued

Compilation of G + C contents of protein coding regions of maize genes		
Protein Class <sup>a</sup>	Range % G + C	Mean % G + C <sup>b</sup>
Uncharacterized Proteins (9)	41.5-70.3	64.3 (+/-7.2)
All Proteins (108)	44.4-75.3	60.8 (+/-5.2) <sup>c</sup>

<sup>a</sup>Number of genes in class given in parentheses.<sup>b</sup>Standard deviations given in parentheses.<sup>c</sup>Combined groups mean ignored in mean calculation

Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of "minor" codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 4. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

34

TABLE 4

Preferred amino acid codons for proteins expressed in maize	
Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG

It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Pat. No. 5,380,831.

## US 10,167,483 B2

35

## 2.2—AAD-12 Plant Rebuild Analysis.

Extensive analysis of the 876 base pairs (bp) of the DNA sequence of the native AAD-12 coding region (SEQ ID NO:1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO:1 (AAD-12) is presented as SEQ ID NO:2. To improve production of the recombinant protein in monocots as well as dicots, a “plant-optimized” DNA sequence AAD-12 (v1) (SEQ ID NO:3) was developed that encodes a protein (SEQ ID NO:4) which is the same as the native SEQ ID NO:2 except for the addition of an alanine residue at the second position (underlined in SEQ ID NO:4). The additional alanine codon (GCT; underlined in SEQ ID

36

NO:3) encodes part of an NcoI restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. Thus, it serves the dual purpose of facilitating subsequent cloning operations while improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized (v1) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native and plant-optimized (v1) DNA sequences of the coding regions are only 79.7% identical. Table 5 shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences (Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

TABLE 5

Codon composition comparisons of coding regions of Native AAD-12, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.

Amino Acid	Codon	A Native #	B Plant Opt v1 #	C Theor. Plant Opt. #
ALA (A)	GCA	1	10	11
	GCC	35	16	15
	GCG	7	0	0
	GCT	0	18	17
ARG (R)	AGA	0	4	5
	AGG	0	4	6
	CGA	0	0	0
	CGC	15	6	4
	CGG	3	0	0
	CGT	0	4	3
ASN (N)	AAC	3	2	2
	AAT	1	2	2
ASP (D)	GAC	15	9	9
	GAT	2	8	8
CYS (C)	TGC	3	2	2
	TGT	0	1	1
END	TAA	1	0	1
	TAG	0	0	
	TGA	0	1	
GLN (Q)	CAA	1	8	7
	CAG	13	6	7
GLU (E)	GAA	3	4	4
	GAG	8	7	7
GLY (G)	GGA	0	8	7
	GGC	24	7	7
	GGG	1	3	4
	GGT	0	7	7
HIS (H)	CAC	8	9	9
	CAT	8	7	7
ILE (I)	ATA	0	2	2
	ATC	10	4	5
	ATT	1	5	4
Totals		163	164	163
Amino Acid	Codon	D Native #	E Plant Opt v1 #	F Theor. Plant Opt. #
LEU (L)	CTA	0	0	0
	CTC	1	8	8
	CTG	23	0	0
	CTT	0	8	8
	TTA	0	0	0
	TTG	0	8	8

US 10,167,483 B2

37

38

TABLE 5 -continued

Codon composition comparisons of coding regions of Native AAD-12, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.				
LYS (K)	AAA	1	1	2
	AAG	5	5	4
MET (M)	ATG	10	10	10
PHE (F)	TTC	7	5	5
	TTT	1	3	3
PRO (P)	CCA	0	5	6
	CCC	9	4	4
	CCG	5	0	0
	CCT	0	5	5
SER (S)	AGC	5	4	3
	AGT	0	0	0
	TCA	0	3	3
	TCC	2	3	3
	TCG	6	0	0
	TCT	0	3	3
THR (T)	ACA	1	4	5
	ACC	11	7	7
	ACG	5	0	0
	ACT	1	7	6
TRP (W)	TGG	8	8	8
TYR (Y)	TAC	4	3	3
	TAT	1	2	2
VAL (V)	GTA	0	0	0
	GTC	6	8	7
	GTG	18	8	9
	GTT	0	8	8
Totals		130	130	130

It is clear from examination of Table 5 that the native and plant-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The Plant-Optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the AAD-12 protein.

### 2.3 Rebuild for *E. coli* Expression

Specially engineered strains of *Escherichia coli* and associated vector systems are often used to produce relatively large amounts of proteins for biochemical and analytical studies. It is sometimes found that a native gene encoding the desired protein is not well suited for high level expression in *E. coli*, even though the source organism for the gene may be another bacterial genus. In such cases it is possible and desirable to reengineer the protein coding region of the gene to render it more suitable for expression in *E. coli*. *E. coli* Class II genes are defined as those that are highly and continuously expressed during the exponential growth phase of *E. coli* cells. (Henaut, A. and Danchin, A. (1996) in *Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology, vol. 2, pp. 2047-2066. Neidhardt, F., Curtiss III, R., Ingraham, J., Lin, E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M. and Umberger, H. (eds.) American Society for Microbiology, Washington, D.C.). Through examination of the codon compositions of the coding regions of *E. coli* Class II genes, one can devise an average codon composition for these *E. coli*-Class II gene

coding regions. It is thought that a protein coding region having an average codon composition mimicking that of the Class II genes will be favored for expression during the exponential growth phase of *E. coli*. Using these guidelines, a new DNA sequence that encodes the AAD-12 protein (SEQ ID NO:4); including the additional alanine at the second position, as mentioned above), was designed according to the average codon composition of *E. coli* Class II gene coding regions. The initial sequence, whose design was based only on codon composition, was further engineered to include certain restriction enzyme recognition sequences suitable for cloning into *E. coli* expression vectors. Detrimental sequence features such as highly stable stemloop structures were avoided, as were intragenic sequences homologous to the 3' end of the 16S ribosomal RNA (i.e. Shine Dalgarno sequences) The *E. coli*-optimized sequence (v2) is disclosed as SEQ ID NO:5 and encodes the protein disclosed in SEQ ID NO:4.

The native and *E. coli*-optimized (v2) DNA sequences are 84.0% identical, while the plant-optimized (v1) and *E. coli*-optimized (v2) DNA sequences are 76.0% identical. Table 6 presents the codon compositions of the native AAD-12 coding region (Columns A and D), an AAD-12 coding region optimized for expression in *E. coli* (v2; Columns B and E) and the codon composition of a theoretical coding region for the AAD-12 protein having an optimal codon composition of *E. coli* Class II genes (Columns C and F).



US 10,167,483 B2

39

40

TABLE 6

Codon composition comparisons of coding regions of Native AAD-12, *E. coli*-Optimized version (v2) and a Theoretical *E. coli* Class II-Optimized version.

Amino Acid	Codon	A Native #	B <i>E. coli</i> Opt v2 #	C Theor. Class II #
ALA (A)	GCA	1	13	13
	GCC	35	0	0
	GCG	7	18	17
	GCT	0	13	14
ARG (R)	AGA	0	0	0
	AGG	0	0	0
	CGA	0	0	0
	CGC	15	6	6
	CGG	3	0	0
	CGT	0	12	12
ASN (N)	AAC	3	4	4
	AAT	1	0	0
ASP (D)	GAC	15	10	9
	GAT	2	7	8
CYS (C)	TGC	3	2	2
	TGT	0	1	1
END	TAA	1	1	1
	TAG	0	0	0
	TGA	0	0	0
GLN (Q)	CAA	1	3	3
	CAG	13	11	11
GLU (E)	GAA	3	8	8
	GAG	8	3	3
GLY (G)	GGA	0	0	0
	GGC	24	12	11
	GGG	1	0	0
	GGT	0	13	14
HIS (H)	CAC	8	11	11
	CAT	8	5	5
ILE (I)	ATA	0	0	0
	ATC	10	7	7
	ATT	1	4	4
Totals		163	164	164
Amino Acid	Codon	D Native #	E <i>E. coli</i> Opt v2 #	F Theor, Class II #
LEU (L)	CTA	0	0	0
	CTC	1	2	0
	CTG	23	20	24
	CTT	0	1	0
	TTA	0	1	0
	TTG	0	0	0
LYS (K)	AAA	1	4	5
	AAG	5	2	1
MET (M)	ATG	10	10	10
PHE (F)	TTC	7	6	6
	TTT	1	2	2
PRO (P)	CCA	0	3	2
	CCC	9	0	0
	CCG	5	11	12
	CCT	0	0	0
SER (S)	AGC	5	4	4
	AGT	0	0	0
	TCA	0	0	0

US 10,167,483 B2

41

42

TABLE 6 -continued

Codon composition comparisons of coding regions of Native AAD-12, *E. coli*-Optimized version (v2) and a Theoretical *E. coli* Class II-Optimized version.

	TCC	2	5	4
	TCG	6	0	0
	TCT	0	4	5
THR (T)	ACA	1	0	0
	ACC	11	12	12
	ACG	5	0	0
	ACT	1	6	6
TRP (W)	TGG	8	8	8
TYR (Y)	TAC	4	3	3
	TAT	1	2	2
VAL (V)	GTA	0	6	6
	GTC	6	0	0
	GTG	18	8	7
	GTT	0	10	11
Totals		130	130	130

It is clear from examination of Table 6 that the native and *E. coli*-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The *E. coli*-Optimized version (v2) closely mimics the codon composition of a theoretical *E. coli*-optimized coding region encoding the AAD-12 protein.

2.4—Design of a Soybean-Codon-Biased DNA Sequence Encoding a Soybean EPSPS Having Mutations that Confer Glyphosate Tolerance.

This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is disclosed as SEQ ID NO:20 of PCT/US2005/014737 (filed May 2, 2005). A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is disclosed as SEQ ID NO:21 of PCT/US2005/014737.

A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (approximately 870 coding sequences), was obtained from the “kazusa.or.jp/codon” World Wide Web site. Those data were reformatted as displayed in Table 7. Columns D and H of Table 7 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 7. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

$$\text{Weighted \% of C1} = 1 / (\% \text{ C1} + \% \text{ C2} + \% \text{ C3} + \text{etc.}) \times \% \text{ C1} \times 100$$

where C1 is the codon in question, C2, C3, etc. represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of Table 7 (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of Table 7. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Ind.).

TABLE 7

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

A Amino Acid	B Codon	C Weighted %	D Soybean %
ALA (A)	GCA	33.1	30.3
	GCC	24.5	22.5
	GCG	<b>DNU*</b>	<b>8.5</b>
	GCT	42.3	38.7

US 10,167,483 B2

43

44

TABLE 7 -continued

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

ARG (R)	AGA	36.0	30.9
	AGG	32.2	27.6
	CGA	<b>DNU</b>	<b>8.2</b>
	CGC	14.8	12.7
	CGG	<b>DNU</b>	<b>6.0</b>
	CGT	16.9	14.5
ASN (N)	AAC	50.0	50.0
	AAT	50.0	50.0
ASP (D)	GAC	38.1	38.1
	GAT	61.9	61.9
CYS (C)	TGC	50.0	50.0
	TGT	50.0	50.0
END	TAA	<b>DNU</b>	40.7
	TAG	<b>DNU</b>	22.7
	TGA	100.0	36.6
GLN (Q)	CAA	55.5	55.5
	CAG	44.5	44.5
GLU (E)	GAA	50.5	50.5
	GAG	49.5	49.5
GLY (G)	GGA	31.9	31.9
	GGC	19.3	19.3
	GGG	18.4	18.4
	GGT	30.4	30.4
HIS (H)	CAC	44.8	44.8
	CAT	55.2	55.2
ILE (I)	ATA	23.4	23.4
	ATC	29.9	29.9
	ATT	46.7	46.7

E Amino Acid	F Codon	G Weighted %	H Soybean %
LEU (L)	CTA	<b>DNU</b>	<b>9.1</b>
	CTC	22.4	18.1
	CTG	16.3	13.2
	CTT	31.5	25.5
	TTA	<b>DNU</b>	<b>9.8</b>
	TTG	29.9	24.2
LYS (K)	AAA	42.5	42.5
	AAG	57.5	57.5
MET (M)	ATG	100.0	100
PHE (F)	TTC	49.2	49.2
	TTT	50.8	50.8
PRO (P)	CCA	39.8	36.5
	CCC	20.9	19.2
	CCG	<b>DNU</b>	<b>8.3</b>
	CCT	39.3	36.0
SER (S)	AGC	16.0	15.1
	AGT	18.2	17.1
	TCA	21.9	20.6
	TCC	18.0	16.9
	TCG	<b>DNU</b>	<b>6.1</b>
	TCT	25.8	24.2
THR (T)	ACA	32.4	29.7
	ACC	30.2	27.7
	ACG	<b>DNU</b>	<b>8.3</b>
	ACT	37.4	34.3
TRP (W)	TGG	100.0	100

US 10,167,483 B2

45

46

TABLE 7 -continued

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.

TYR (Y)	TAC	48.2	48.2
	TAT	51.8	51.8
VAL (V)	GTA	11.5	11.5
	GTC	17.8	17.8
	GTG	32.0	32.0
	GTT	38.7	38.7

\*DNU = Do Not Use

To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 from PCT/US2005/014737 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21 of PCT/US2005/014737.

The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is disclosed as bases 1-1575 of SEQ ID NO:22 of PCT/US2005/014737. Synthesis of a DNA fragment comprising SEQ ID NO:22 of PCT/US2005/014737 was performed by a commercial supplier (PicoScript, Houston Tex.).

### Example 3—Cloning of Expression and Transformation Vectors

#### 3.1 Construction of *E. coli* pET Expression Vector.

Using the restriction enzymes corresponding to the sites added with the additional cloning linkers (Xba I, Xho I) AAD-12 (v2) was cut out of the picoscript vector, and ligated into a pET280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP 10F' *E. coli*, and plated on to Luria Broth+50 µg/ml Streptomycin & Spectinomycin (LB S/S) agar plates.

To differentiate between AAD-12 (v2): pET280 and pCR2.1: pET280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB-S/S, and grown at 37° C. for 4 hours with agitation. Each culture was then spotted onto LB+Kanamycin 50 µg/ml plates, which were incubated at 37° C. overnight. Colonies that grew on the LB-K were

assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before, and checked for correctness with digestion by XbaI/XhoI. The final expression construct was given the designation pDAB3222.

#### 3.2—Construction of *Pseudomonas* Expression Vector

The AAD-12 (v2) open reading frame was initially cloned into the modified pET expression vector (Novagen), “pET280 S/S”, as an XbaI-XhoI fragment. The resulting plasmid pDAB725 was confirmed with restriction enzyme digestion and sequencing reactions. The AAD-12 (v2) open reading frame from pDAB725 was transferred into the *Pseudomonas* expression vector, pMYC1803, as an XbaI-XhoI fragment. Positive colonies were confirmed via restriction enzyme digestion. The completed construct pDAB739 was transformed into the MB217 and MB324 *Pseudomonas* expression strains.

#### 3.3—Completion of Binary Vectors.

The plant optimized gene AAD-12 (v1) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:3) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:6) and M13 Reverse (SEQ ID NO:7) primers using the Beckman Coulter “Dye Terminator Cycle Sequencing with Quick Start Kit” reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized AAD-12 (v1) DNA sequence. The AAD-12 (v1) gene was cloned into pDAB726 as an Nco I-Sac I fragment. The resulting construct was designated pDAB723, containing: [AtUbi10 promoter: Nt OSM 5'UTR: AAD-12 (v1): Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with a PvuII and a Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB724, containing the following cassette [AtUbi10 promoter Nt OSM5'UTR: AAD-12 (v1): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter PAT: ORF25/26 3'UTR] was restriction digested (with Bam HI, Nco I, Not I, SacI, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB724) was used for transformation into *Agrobacterium* (see section 7.2).

#### 3.4—Cloning of Additional Transformation Constructs.

All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular cloning methods (Maniatis et al., 1982). Table 8 lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

US 10,167,483 B2

47

48

TABLE 8

Binary constructs used in transformation of various plant species.													
pDAB #	pDAS #	Species Trans-formed into *	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Promoter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
724	—	A, Ct, S	AAD12 v1	AtUbi10	NtOsm	—	—	—	Erythro-mycin	—	pat	CsVMV	Agro binary
3274	—	A	AAD12 v1	AtUbi10	NtOsm	RB7 Mar v2	—	—	Specti-nomycin	—	—	—	Agro binary
3278	1580	T	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	—	—	Specti-nomycin	—	pat	AtUbi10	Agro binary
3285	—	A	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	—	—	Specti-nomycin	—	pat	AtUbi10	Agro binary
3759	—	A, Ca, S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Specti-nomycin	—	pat	AtUbi10	Agro binary
4101	1863	Cn, R	AAD12 v1	ZmUbi1	—	RB7 Mar v2	—	—	Ampicillin	—	AHAS v3	OsAct1	Whiskers/ Gun
4464	—	S	AAD12 v1	CsVMV	—	RB7 Mar v2	—	—	Specti-nomycin	—	pat	CsVMV	Agro binary
4468	—	S	AAD12 v1	AtUbi10	—	RB7 Mar v2	—	—	Specti-nomycin	—	pat	CsVMV	Agro binary
4472	—	S	AAD12 v1	AtUbi3	—	RB7 Mar v2	—	—	Specti-nomycin	—	pat	CsVMV	Agro binary
4476	—	S	AAD12 v1	ZmUbi1	—	RB7 Mar v2	—	—	Specti-nomycin	—	pat	CsVMV	Agro binary
4480	—	S	AAD12 v1	AtAct2	—	RB7 Mar v2	—	—	Specti-nomycin	—	pat	CsVMV	Agro binary

\* A = *Arabidopsis* CsVMV = Cassava Vein Mosaic Virus Promoter ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter T = Tobacco AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter HptII = hygromycin phosphotransferase S = Soybean AtUbi3 = *Arabidopsis thaliana* Ubiquitin 3 Promoter Ct = Cotton AtAct2 = *Arabidopsis thaliana* Actin 2 Promoter R = Rice RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR) Cn = Corn Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region Ca = Canola

#### Example 4—Recombinant AAD-12 (v2) Expression and Purification in *Pseudomonas fluorescens*

##### 4.1—*Pseudomonas fluorescens* Fermentation

For shake flask experiment, 200 µl of *Pseudomonas fluorescens* strain MB324 glycerol stock carried AAD-12 (v2) construct pDAB739 (sec 3.2) was used to inoculate 50 ml fresh LB media supplemented with 30 µg/ml tetracycline/HCl. The culture (in a 250 ml baffled Erlenmeyer flask) was incubated on a shaker (New Brunswick Scientific Model Innova 44) at 300 rpm and 30° C. for 16 hrs. 20 ml of seed culture was transferred into 1 L *Pseudomonas fluorescens* culture media (Yeast extract, 5 g/L; K<sub>2</sub>HPO<sub>4</sub>, 5 g/L; (NH<sub>4</sub>)<sub>2</sub>PO<sub>4</sub>, 7.5 g/L; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 g/L; KCl, 0.5 g/L; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 g/L; NaCitrate·2H<sub>2</sub>O, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 ml/L; Trace element solution: FeCl<sub>3</sub>·6H<sub>2</sub>O, 5.4 g/L; MnCl<sub>2</sub>·4H<sub>2</sub>O, 1 g/L; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1.45 g/L; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.25 g/L; H<sub>3</sub>BO<sub>3</sub>, 0.1 g/L; (NH<sub>4</sub>)<sub>6</sub>MO<sub>7</sub>O<sub>24</sub>, 0.1 g/L; concentrated HCl, 13 ml/L) supplemented with 20 µg/ml tetracycline/HCl and 250 µl of Pluronic L61(anti-foam) in a 2.8 L baffled Erlenmeyer flask. The cultures were incubated at 30° C. and 300 rpm for 24 hrs. Isopropyl β-D-1-thiogalacto-pyranoside (IPTG) was added to 1 mM final in the cultures and continued to incubate for approximately 48 hrs at 25° C. Cells were harvested by centrifugation at 7 krpm at 4° C. for 15 min, and cell paste was stored at -80° C. or immediately processed for purification.

For tank experiments, 1 ml each of the glycerol stock was inoculated a 1 L baffled flask containing 200 ml of LB media supplemented with 30 µg/ml tetracycline/HCl at 300 rpm and 32° C. for 16-24 hrs. The combined culture from three flasks (600 ml) was then aseptically transferred to a 20 L fermentor (B. Braun Bioreactor Systems) containing 10 L of Dow proprietary defined medium (through Teknova, Hollister, Calif.) designed to support high cell density growth. Growth temperature was maintained at 32° C. and the pH was controlled at the desired set-point through the addition of aqueous ammonia. Dissolved oxygen was maintained at a positive level in the liquid culture by regulating the sparged air flow and the agitation rates. The fed-batch fermentation process was carried out for approximately 24 hrs till cell density reached 170-200 OD<sub>575</sub>. IPTG was added to 1 mM to induce the recombinant protein expression and the temperature was reduced and maintained to 25° C. using circulation of cold-water supply. The induction phase of the fermentation was allowed to continue for another 24 hrs. Samples (30 ml) were collected for various analyses to determine cell density and protein expression level at 6, 12, and 18 hrs post-induction time points. At the end of a fermentation run, cells were harvested by centrifugation at 10 krpm for 30 min. The cell pellets were frozen at -80° C. for further processing.

##### 4.2—Purification of AAD-12 (v2) for Biochemical Characterization and Antibody Production

Approximately 100-200 g of frozen (or fresh) *Pseudomonas* cells were thawed and resuspended in 1-2 L of extraction

US 10,167,483 B2

49

buffer containing 20 mM Tris-HCl, pH 8.5, and 25 ml of Protease inhibitor cocktail (Sigma cat#P8465). The cells were disrupted using Microfluidizer (model M110L or 110Y) (Microfluidics, Newton, Mass.) on ice with one pass at 11,000-12,000 psi. The lysate was centrifuged at 24,000 rpm for 20 min. The supernatant was transferred and dialyzed against 10 volumes of 20 mM Tris-HCl, pH 8.5 overnight at 4° C., or diafiltrated with this buffer and filtered through a 0.45 µm membrane before applying to the column separations. All subsequent protein separations were performed using Pharmacia AKTA Explorer 100 and operated at 4° C. Prior to loading, a Q Sepharose Fast Flow column (Pharmacia XK 50/00, 500 ml bed size) was equilibrated with 20 mM Tris-HCl, pH 8.5 buffer. The sample was applied to the column at 15 ml/min and then washed with this buffer until the eluate OD<sub>280</sub> returned to baseline. Proteins were eluted with 2 L of linear gradient from 0 to 0.3 M NaCl at a flow rate of 15 ml/min, while 45 ml fractions were collected. Fractions containing AAD-12 activity as determined by the colorimetric enzyme assay and also corresponding to the predicted molecular weight of AAD-12 protein (about 32 kDa band on SDS-PAGE) were pooled. Solid ammonium sulfate to final 0.5 M was added to the sample, and then applied to a Phenyl HP column (Pharmacia XK 50/20, 250 ml bed size) equilibrated in 0.5 M ammonium sulfate in 20 mM Tris-HCl, pH 8.0. This column was washed with the binding buffer at 10 ml/min until the OD<sub>280</sub> of the eluate returned to baseline, proteins were eluted within 2 column volumes at 10 ml/min by a linear gradient from 0.5 M to 0 M Ammonium sulfate in 20 mM Tris-HCl, pH 8.0, and 12.5 ml fractions were collected. The main peak fractions containing AAD-12 were pooled, and if necessary, concentrated using a MWCO 10 kDa cut-off membrane centrifugal filter device (Millipore). In some cases the sample was further applied to a Superdex 75 gel filtration column (Pharmacia XK 16/60, 110 ml bed size) with PBS buffer at a flow rate of 1 ml/min. Peak fractions containing pure AAD-12 were pooled and stored at -80° C. In most cases, AAD-12 protein purity is approaching or above 99% after sequential ion-exchange column and hydrophobic interaction column two-step separation. A typical yield for purified AAD-12 is 12-18 mg/g of wet cells. Bulk protein sample was formulated in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM DTT, and 1% Trehalose by diafiltration, and lyophilized on the Virtis Freezemobile Model 25EL (Virtis, Cardiner, N.Y.) for long-term storage.

Protein concentration was initially measured by Bradford assay using Bio-Rad Protein assay kit (cat#500-0006) with bovine serum albumin as standard. When needed, more accurate protein concentration was determined by using total amino acid hydrolysis. The sample was analyzed in Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, Calif.) with amino acid calibration standards (cat#PN5061-3330) purchased from Agilent.

AAD-12 activity was determined through out the processes to ensure no loss of the enzyme activity by each treatment and manipulation, as described in the Example 5 below. Protein purity was monitored by using SDS-PAGE and analytical size exclusion chromatography. Purified protein sample was further verified and confirmed by N-terminal amino acid sequencing, and shown consisting of expected AQTTLQITPT residues at its N-terminus. Short and long-term protein stability was tested by enzymatic activity and by native-PAGE and SDS-PAGE gel analysis under both non-reducing and reducing conditions. And it was noticed that AAD-12 is prone to oligomerization via disulfide bond formation, therefore typically 2 mM DTT was

50

used for protein storage. Phosphate-buffer saline (PBS) and Tris-buffer saline (TBS) were tested for protein lyophilization, with and without the presence of 1% trehalose. Additionally, the endotoxin and DNA contaminant context from purified sample were measured respectively, and the integrity of the AAD-12 protein was also assessed by isoelectric focusing (IEF) analysis.

Ten milligrams of purified AAD-12 (v2) was delivered to Zymed Laboratories, Inc. (South San Francisco, Calif.) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 ml of complete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification, and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

#### Example 5—In Vitro Assays of AAD-12 Activity

##### 5.1—Assay Via Colorimetric Phenol Detection.

Enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxygenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100 µM in 0.15 ml 20 mM MOPS pH 6.75 containing 200 µM NH<sub>4</sub>(FeSO<sub>4</sub>)<sub>2</sub>, 200 µM sodium ascorbate. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlorophenol was linear and proportional to the concentration of phenol in the assay up to ~500 µM. A calibration curve performed under standard assay conditions (160 µl final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 17.2 µM phenol.

Enzyme assays were performed in a total volume of 0.16 ml 20 mM MOPS pH 6.75 containing 200 µM NH<sub>4</sub>FeSO<sub>4</sub>, 200 µM sodium ascorbate, 1 mM α-ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or α-ketoglutarate at time zero. After 5 minutes of incubation at 25° C., the reaction was terminated by addition of 30 µl of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer (3.09 g boric acid+3.73 g KCl+44 ml 1 N KOH) and 0.2% 4-aminoantipyrine. Then 10 µl 0.8% potassium ferricyanide was added and after 5 or 10 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols.

##### 5.2—Assay Via Detection of Chloropyridinol

AAD-12 action on potential substrates such as the herbicide triclopyr containing a substituted pyridine (rather than benzene rings) will release a pyridinol on cleavage of the aryloxyalkanoate bond. Pyridinols were not detected using the aminoantipyrine/ferricyanide phenol detection described in the preceding section. However, it was found that product chloropyridinols absorb strongly in the near UV with λ<sub>max</sub> of 325 nm at pH 7 (extinction coefficient ~8,400 M<sup>-1</sup>·cm<sup>-1</sup>). This was used to create a continuous microplate-based spectrophotometric assay. Assays were performed in a total



US 10,167,483 B2

## 51

volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200  $\mu$ M  $\text{NH}_4\text{FeSO}_4$ , 200  $\mu$ M sodium ascorbate, 1 mM  $\alpha$ -ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or  $\alpha$ -ketoglutarate at time zero and the increase in absorbance followed for 10 minutes at 325 nm in a microplate reader. The first 2 minutes of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200  $\mu$ l final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9  $\mu$ M chlorpyridinol.

### 5.3—Colorimetric Assay Using 2-(2-chloro,4-nitrophenoxy)propionate

A convenient assay of AAD-12 was devised using 2-(2-chloro,4-nitrophenoxy)propionate (CNPP) as substrate. Cleavage of CNPP by AAD-12 releases 2-chloro,4-nitrophenol. This phenol has a bright yellow absorbance at 410 nm at pH 7 enabling the reaction to be followed continuously or by endpoint analysis. The presence of AAD-12 activity can be monitored visually without the need for addition of further reagents. Microplate-based spectrophotometric assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200  $\mu$ M  $\text{NH}_4\text{FeSO}_4$ , 200  $\mu$ M sodium ascorbate, 1 mM  $\alpha$ -ketoglutarate, the appropriate amount of CNPP (added from a 10 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of CNPP, enzyme, or  $\alpha$ -ketoglutarate at time zero and the increase in absorbance followed for 10 min at 410 nm in a microplate reader. The first 2 min of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200  $\mu$ l final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1  $\mu$ M 2-chloro, 4-nitrophenol. Using this assay, the kinetic constants for CNPP as a substrate were determined to be  $K_m = 31 \pm 5.5$   $\mu$ M and  $k = 16.2 \pm 0.79$   $\text{min}^{-1}$ .

### Example 6—In Vitro Activity of AAD-12 on Various Substrates

#### 6.1—AAD-12 (v2) activity on (R,S)-dichlorprop, (R)-dichlorprop, (S)-dichlorprop and 2,4-D

Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4  $\mu$ g purified AAD-12 (v2). (R,S)-dichlorprop (R,S-DP) was tested at 1 mM and (R)-dichlorprop, (S)-dichlorprop and 2,4-D were tested at 0.5 mM. The results are shown in FIG. 3, which illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop. 4.4  $\mu$ g AAD-12 (v2) was incubated with 0.5 mM substrate (1 mM for (R,S)-dichlorprop) and the reaction initiated by addition of  $\alpha$ -ketoglutarate. After 5 minutes, the reaction was quenched, and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.

AAD-12 (v2) has excellent activity on (R,S)-dichlorprop and (S)-dichlorprop and has minimal activity on (R)-dichlorprop. This indicates that AAD-12 (v2) has a clear (S)-enantiomeric preference. The activity of AAD-12 (v2) on 2,4-D was equivalent to that on (S)-dichlorprop indicating that the enzyme can process oxypropionate and oxyacetates effectively.

#### 6.2—AAD-12 (v2) Activity on Pyridyloxyalkanoates

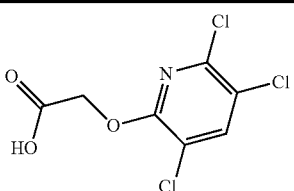
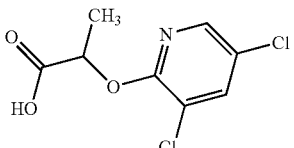
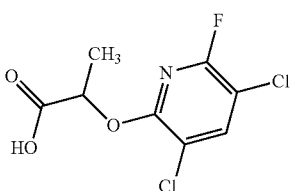
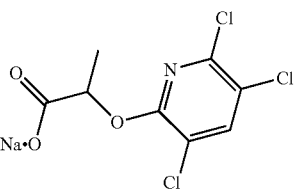
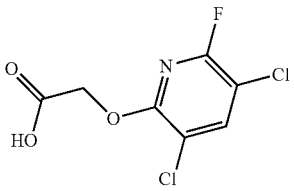
Using the pyridinol assay described in Example 5.2, five pyridyloxyalkanoates were assayed at 1 mM in a reaction mix containing 6.8  $\mu$ g purified AAD-12 (v2). The rates of each reaction were monitored and are presented in Table 9.

## 52

All five pyridyloxyalkanoates were cleaved to release pyridinols by AAD-12 (v2). The rates for the oxypropionate substrates 116844 and 91767 were somewhat faster than those for the corresponding acetates (triclopyr and 93833 respectively) indicating a preference of AAD-12 (v2) for oxypropionate over oxyacetate side chains. These data show that AAD-12 (v2) is able to effectively degrade pyridyloxyalkanoate herbicides such as triclopyr.

TABLE 9

Rates of pyridyloxyalkanoate cleavage by AAD-12 (v2). 6.8  $\mu$ g AAD-12 (v2) was incubated with 1 mM substrate, the reaction initiated by addition of  $\alpha$ -ketoglutarate and the subsequent increase in absorbance monitored at 325 nm. The background rate of 1.4 mAU/min without  $\alpha$ -ketoglutarate was subtracted from the rates with substrate.

STRUCTURE	ID	Rate (mAU/min)	Rate relative to triclopyr
	triclopyr	97	1
	66357	225	2.3
	91767	190	0.8
	116844	257	1.4
	93833	118	0.5

#### 6.3—Kinetic Constants of AAD-12 (v2) for 2,4-D, (R,S)-DCP and Triclopyr

The  $K_m$  and  $k_{cat}$  values of purified AAD-12 (v2) for the herbicides 2,4-D, (R,S)-dichlorprop and triclopyr were determined using the appropriate assay method. Substrate inhibition occurred at high concentrations (>1 mM) of 2,4-D and (R,S)-DCP so concentrations below this were used to fit the data to the Michaelis-Menten equation using Grafit 4.0 (Erithacus Software, UK). No substrate inhibition was noted for triclopyr up to 2 mM. The kinetic constants are summa-

rized in Table 10. From these data, the rate of AAD-12 (v2) cleavage of triclopyr is ~5% that of 2,4-D, under maximal velocity conditions.

TABLE 10

Kinetic constants of AAD-12 (v2) for three herbicide substrates				
Substrate	$K_m$ , $\mu\text{M}$ ( $\pm\text{SE}$ )	$k_{cat}$ , $\text{min}^{-1}$ ( $\pm\text{SE}$ )	Assay method	Substrate inhibition at 2 mM
2,4-D	102 ( $\pm 18.4$ )	54.1 ( $\pm 3.1$ )	Phenol detection	55%
(R,S)-dichlorprop	122 ( $\pm 2.7$ )*	63.4 ( $\pm 0.5$ )	Phenol detection	55%
Triclopyr	241 ( $\pm 30$ )	2.6 ( $\pm 0.1$ )	AA325 nm	0%

\*Because of the (S)-enantiomeric preference of AAD-12, the  $K_m$  value was calculated assuming 50% of the racemic mixture was available as a substrate

#### Example 7—Transformation into *Arabidopsis* and Selection

##### 7.1—*Arabidopsis thaliana* Growth Conditions.

Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, Mo.) solution. The suspended seed was stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, Wash.) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150  $\mu\text{mol}/\text{m}^2$  sec under constant temperature (22° C.) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

##### 7.2—*Agrobacterium* Transformation.

An LB+agar plate with erythromycin (Sigma Chemical Co., St. Louis, Mo.) (200 mg/L) or spectinomycin (100 mg/L) containing a streaked DH5 $\alpha$  colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB+erythromycin). The cultures were incubated overnight at 37° C. with constant agitation. Qiagen (Valencia, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50  $\mu\text{l}$  of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions, Voltage: 2.4 kV, Pulse length: 5 msec.

After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C. in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP+agar with eryth-

romycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, Mo.) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

Colonies were selected and streaked onto fresh YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28° C. for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP+erythromycin (200 mg/L) or spectinomycin (100 mg/L)) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed *Agrobacterium* cells, suspended in 10  $\mu\text{l}$  of water, at 100° C. for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wis.) per manufacturer's instructions at 0.5 $\times$  concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C. for 3 minutes, 2) 94° C. for 45 seconds, 3) 55° C. for 30 seconds, 4) 72° C. for 1 minute, for 29 cycles then 1 cycle of 72° C. for 10 minutes. The reaction was maintained at 4° C. after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

##### 7.3—*Arabidopsis* Transformation.

*Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C. with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C. with constant agitation. The cells were then pelleted at approx. 8700 $\times$ g for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing:  $\frac{1}{2}\times$  Murashige and Skoog salts/Gamborg's B5 vitamins, 10% (w/v) sucrose, 0.044  $\mu\text{M}$  benzylamino purine (10  $\mu\text{l}$ /liter of 1 mg/ml stock in DMSO) and 300  $\mu\text{l}$ /liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C., with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

##### 7.4—Selection of Transformed Plants.

Freshly harvested T<sub>1</sub> seed [AAD-12 (v1) gene] was allowed to dry for 7 days at room temperature. T<sub>1</sub> seed was sown in 26.5 $\times$ 51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T<sub>1</sub> seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) was covered with fine vermiculite and subirrigated

with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed PAT gene).

Seven days after planting (DAP) and again 11 DAP, T<sub>1</sub> plants (cotyledon and 2-4-1f stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22±5° C., 50±30% RH, 14 h light:10 dark, minimum 500 µE/m<sup>2</sup>s<sup>-1</sup> natural+supplemental light) at least 1 day prior to testing for the ability of AAD-12 (v1) (plant optimized gene) to provide phenoxy auxin herbicide resistance.

T<sub>1</sub> plants were then randomly assigned to various rates of 2,4-D. For *Arabidopsis*, 50 g ae/ha 2,4-D is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (50, 200, 800, or 3200 g ae/ha). Tables 10 and 11 show comparisons drawn to an aryloxyalkanoate herbicide resistance gene (AAD-1 (v3)) previously described in PCT/US2005/014737.

All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.). Dichlorprop used was commercial grade formulated as potassium salt of R-dichlorprop (600 g ai/L, AH Marks). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of herbicides in 200 mM HEPES buffer, pH 7.5.

Some T<sub>1</sub> individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether the pyridyloxyacetate auxin herbicides, triclopyr and fluroxypyr, could be effectively degraded in planta. Herbicides were applied to T<sub>1</sub> plants with use of a track sprayer in a 187 L/ha spray volume. T<sub>1</sub> plants that exhibited tolerance to 2,4-D DMA were further accessed in the T<sub>2</sub> generation.

#### 7.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-12 (v1) (plant optimized gene). T<sub>1</sub> transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T<sub>1</sub> seed were screened and 316 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.10% which lies in the normal range of selection frequency of constructs where PAT+Liberty are used for selection. T<sub>1</sub> plants selected above were subse-

quently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 11 compares the response of AAD-12 (v1) and control genes to impart 2,4-D resistance to *Arabidopsis* T<sub>1</sub> transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-400/), or severe injury (>40%). Since each T<sub>1</sub> is an independent transformation event, one can expect significant variation of individual T<sub>1</sub> responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The AAD-12 (v1) gene imparted herbicide resistance to individual T<sub>1</sub> *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 11 simply to demonstrate the significant difference between the plants transformed with AAD-12 (v1) versus the wildtype or PAT/Cry1F-transformed controls. Injury levels tend to be greater and the frequency of uninjured plants was lower at elevated rates up to 3,200 g ae/ha (or 6× field rate). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, many individuals have survived 3,200 g ae/ha 2,4-D with little or no injury.

TABLE 11

AAD-12 (v1) transformed T<sub>1</sub> *Arabidopsis* response to a range of 2,4-D rates applied postemergence, compared to an AAD-1 v3 (T<sub>4</sub>) homozygous resistant population, or a Pat-Cry1F transformed, auxin-sensitive control.

	<u>% Injury</u>			% Injury	Std
	<20%	20-40%	>40%	Ave	Dev
AAD-12 (v1) gene					
T <sub>1</sub> transformants					
<u>Averages</u>					
Untreated control-buffer	6	0	0	0	0
50 g ae/ha 2,4-D	6	0	2	16	24
200 g ae/ha 2,4-D	6	1	1	11	18
800 g ae/ha 2,4-D	5	2	1	15	20
3200 g ae/ha 2,4-D	8	0	0	6	6
PAT/Cry1F					
(transformed control)					
<u>Averages</u>					
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	4	1	5	31	16
200 g ae/ha 2,4-D	0	0	10	70	2
800 g ae/ha 2,4-D	0	0	10	81	8
3200 g ae/ha 2,4-D	0	0	10	91	2
Homozygous AAD-1					
(v3) gene T <sub>4</sub> plants					
<u>Averages</u>					
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	10	0	0	0	0
200 g ae/ha 2,4-D	10	0	0	0	0
800 g ae/ha 2,4-D	10	0	0	0	0
3200 g ae/ha 2,4-D	9	1	0	2	6

US 10,167,483 B2

57

Table 12 shows a similarly conducted dose response of *T*<sub>1</sub> *Arabidopsis* to the phenoxypropionic acid, dichlorprop. The data shows that the herbicidally active (R-) isomer of dichlorprop does not serve as a suitable substrate for AAD-12 (v1). The fact that AAD-1 will metabolize R-dichlorprop well enough to impart commercially acceptable tolerance is one distinguishing characteristic that separates the two genes. (Table 12). AAD-1 and AAD-12 are considered R- and S-specific  $\alpha$ -ketoglutarate dioxygenases, respectively.

TABLE 12

<i>T</i> <sub>1</sub> <i>Arabidopsis</i> response to a range of R-dichlorprop rates applied postemergence.					
	% Injury			% Injury	Std Dev
	<20%	20-40%	>40%	Ave	
AAD-12 v1 gene Averages					
Untreated control	6	0	0	0	0
50 g ae/ha R-dichlorprop	0	0	8	63	7
200 g ae/ha R-dichlorprop	0	0	8	85	10
800 g ae/ha R-dichlorprop	0	0	8	96	4
3200 g ae/ha R-dichlorprop	0	0	8	98	2
PAT/Cry1F Averages					
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	0	10	0	27	2
200 g ae/ha R-dichlorprop	0	0	10	69	3
800 g ae/ha R-dichlorprop	0	0	10	83	6
3200 g ae/ha R-dichlorprop	0	0	10	90	2
Homozygous AAD-1 (v3) gene <i>T</i> <sub>4</sub> plants					
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	10	0	0	0	0
200 g ae/ha R-dichlorprop	10	0	0	0	0
800 g ae/ha R-dichlorprop	10	0	0	0	0
3200 g ae/ha R-dichlorprop	10	0	0	0	0

#### 7.6—AAD-12 (v1) as a Selectable Marker.

The ability to use AAD-12 (v1) as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed as described above. Approximately 50 *T*<sub>4</sub> generation *Arabidopsis* seed (homozygous for AAD-12 (v1)) were spiked into approximately 5,000 wild-type (sensitive) seed. Several treatments were compared, each tray of plants receiving either one or two application timings of 2,4-D in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 followed by 11 DAP. Since all individuals also contained the PAT gene in the same transformation vector, AAD-12 selected with 2,4-D could be directly compared to PAT selected with glufosinate.

Treatments were applied with a DeVilbiss spray tip as previously described. Plants were identified as Resistant or Sensitive 17 DAP. The optimum treatment was 75 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), was equally effective in selection frequency, and resulted in less herbicidal injury to the transformed individuals than the Liberty selection scheme. These results indicate AAD-12 (v1) can be effectively used as an alternative selectable marker for a population of transformed *Arabidopsis*.

#### 7.7—Heritability.

A variety of *T*<sub>1</sub> events were self-pollinated to produce *T*<sub>2</sub> seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random *T*<sub>2</sub> siblings. Each individual *T*<sub>2</sub> plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). Seventy-five percent of the *T*<sub>1</sub> families (*T*<sub>2</sub> plants) segre-

58

gated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis ( $P>0.05$ ).

Seed were collected from 12 to 20 *T*<sub>2</sub> individuals (*T*<sub>3</sub> seed). Twenty-five *T*<sub>3</sub> siblings from each of eight randomly-selected *T*<sub>2</sub> families were progeny tested as previously described. Approximately one-third of the *T*<sub>2</sub> families anticipated to be homozygous (non-segregating populations) have been identified in each line. These data show AAD-12 (v1) is stably integrated and inherited in a Mendelian fashion to at least three generations.

#### 7.8—Additional Foliar Applications Herbicide Resistance in AAD-12 *Arabidopsis*.

The ability of AAD-12 (v1) to provide resistance to other aryloxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates. *T*<sub>2</sub> generation *Arabidopsis* seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed with a range of pyridyloxyacetate herbicides: 280-2240 g ae/ha triclopyr (Garlon 3A, Dow AgroSciences) and 280-2240 g ae/ha fluroxypyr (Starane, Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-12 activity, 2,4-dichlorophenol (DCP, Sigma) (at a molar equivalent to 280-2240 g ae/ha of 2,4-D, technical grade DCP was used). All applications were formulated in water. Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment.

There is no effect of the 2,4-D metabolite, 2,4-dichlorophenol (DCP), on transgenic non-AAD-12 control *Arabidopsis* (Pat/Cry1F). AAD-12-transformed plants were also clearly protected from the triclopyr and fluroxypyr herbicide injury that was seen in the transformed non-resistant controls (see Table 13). These results confirm that AAD-12 (v1) in *Arabidopsis* provides resistance to the pyridyloxyacetic auxins tested. This is the first report of an enzyme with significant activity on pyridyloxyacetic acid herbicides. No other 2,4-D degrading enzyme has been reported with similar activity.

TABLE 13

Comparison of <i>T</i> <sub>2</sub> AAD-12 (v1) and transformed control <i>Arabidopsis</i> plant response to various foliar-applied auxinic herbicides.			
Herbicide Treatment	Ave % Injury 14DAT		
	Segregating <i>T</i> <sub>2</sub> AAD-12 (v1) plants (pDAB724.01.120)	Pat/Cry1F Control	
Pyridyloxyacetic auxins			
280 g ae/ha Triclopyr	0	52	
560 g ae/ha Triclopyr	3	58	
1120 g ae/ha Triclopyr	0	75*	
2240 g ae/ha Triclopyr	3	75*	
280 g ae/ha Fluroxypyr	0	75*	
560 g ae/ha Fluroxypyr	2	75*	
1120 g ae/ha Fluroxypyr	3	75*	
2240 g ae/ha Fluroxypyr	5	75*	
Inactive DCP metabolite			
280 g ae/ha 2,4-DCP	0	0	
560 g ae/ha 2,4-DCP	0	0	



US 10,167,483 B2

59

TABLE 13-continued

Comparison of T <sub>2</sub> AAD-12 (v1) and transformed control <i>Arabidopsis</i> plant response to various foliar-applied auxinic herbicides.		
Ave % Injury 14DAT		
Herbicide Treatment	Segregating T <sub>2</sub> AAD-12 (v1) plants (pDAB724.01.120)	Pat/Cry1f-Control
1120 g ae/ha 2,4-DCP	0	0
2240 g ae/ha 2,4-DCP	0	0

\*Plants in this experiment were stunted and severely epinastic, but remained green and did not receive injury ratings >75%.

### 7.9—Molecular Analysis of AAD-12 (v1) *Arabidopsis*.

Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple AAD-12 (v1) homozygous lines to determine stable integration of the plant transformation unit containing PAT and AAD-12 (v1). Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

Results showed that all 2,4-D resistant plants assayed, contained PAT (and thus by inference, AAD-12 (v1)). Copy number analysis showed total inserts ranged from 1 to 5 copies. This correlates, too, with the AAD-12 (v1) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance to all commercially available phenoxyacetic and pyridyloxyacetic acids.

### 7.10—*Arabidopsis* Transformed with Molecular Stack of AAD-12 (v1) and a Glyphosate Resistance Gene.

T<sub>1</sub> *Arabidopsis* seed was produced, as previously described, containing the pDAB3759 plasmid (AAD-12 (v1)+EPSPS) which encodes a putative glyphosate resistance trait. T<sub>1</sub> transformants were selected using AAD-12 (v1) as the selectable marker as described in example 7.6. T<sub>1</sub> plants (individually transformed events) were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, AAD-12 (v1)+PAT T<sub>4</sub> homozygous lines (pDAB724-transformed), and PAT+Cry1F homozygous line (transformed control). The pDAB3759 and pDAB724 transformed plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in water. All treatments were replicated 5 to 20 times. Plants were evaluated 7 and 14 days after treatment.

Initial resistance assessment indicated plants tolerant to 2,4-D were subsequently tolerant to glyphosate when compared to the response of the three control lines. These results indicate that resistance can be imparted to plants to two herbicides with differing modes of action, including 2,4-D and glyphosate tolerance, allowing application of both herbicides postemergence. Additionally, AAD-12+2,4-D was used effectively as a selectable marker for a true resistance selection.

60

TABLE 14

T <sub>1</sub> <i>Arabidopsis</i> response to a range of glyphosate rates applied postemergence (14 DAT).					
	% Injury			% Injury	
	<20%	20-40%	>40%	Ave	Std Dev
AAD-12 v1 gene + EPSPS + HptII (pDAB3759) (Averages)					
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	13	2	1	11	16
105 g ae/ha glyphosate	10	1	5	34	38
420 g ae/ha glyphosate	5	6	5	44	37
1680 g ae/ha glyphosate	0	0	16	85	9
PAT/Cry1F					
Averages					
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	67	7
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0
Wildtype (Col-0)					
Averages					
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	75	13
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0
pDAB724 T <sub>4</sub> (PAT + AAD-12)					
Averages					
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	66	8
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0

### 7.11—AAD-12 *Arabidopsis* Genetically Stacked with AAD-1 to Give Wider Spectrum of Herbicide Tolerance.

AAD-12 (v1) (pDAB724) and AAD-1 (v3) (pDAB721) plants were reciprocally crossed and F<sub>1</sub> seed was collected. Eight F<sub>1</sub> seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F<sub>1</sub> plants and subjected to Western analysis to confirm the presence of both genes. It was concluded that all 8 plants tested expressed both AAD-1 and AAD-12 proteins. The seed was bulked and allowed to dry for a week before planting.

One hundred F<sub>2</sub> seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F<sub>2</sub> plants survived glufosinate selection fitting an expected segregation ratio for two independently assorting loci for glufosinate resistance (15R: 1S). Glufosinate resistant plants were then treated with 560 g ae/ha R-dichlorprop+560 g ae/ha triclopyr, applied to the plants under the same spray regimen as used for the other testing. Plants were graded at 3 and 14 DAT. Sixty-three of the 96 plants that survived glufosinate selection also survived the herbicide application. These data are consistent with an expected segregation pattern (9R: 6S) of two independently assorting dominant traits where each gene gives resistance to only one of the auxinic herbicides (either R-dichlorprop or triclopyr). The results indicate that AAD-12 (pDAB724) can be successfully stacked with AAD-1 (pDAB721), thus increasing the spectrum herbicides that may be applied to the crop of interest [(2,4-D+R-dichlorprop) and (2,4-D+fluroxypyr+triclopyr), respectively]. This could be useful to bring 2,4-D tolerance to a very sensitive species through conventional stacking of two separate 2,4-D resistance genes. Additionally, if either gene were used as a selectable marker for a third and fourth gene of interest

US 10,167,483 B2

61

through independent transformation activities, then each gene pair could be brought together through conventional breeding activities and subsequently selected in the F<sub>1</sub> generation through paired sprays with herbicides that are exclusive between the AAD-1 and AAD-12 enzymes (as shown with R-dichlorprop and triclopyr for AAD-1 and AAD-12, respectively, above).

Other AAD stacks are also within the scope of the subject invention. The TfdA protein discussed elsewhere herein (Streber et al.), for example, can be used together with the subject AAD-12 genes to impart novel spectrums of herbicide resistance in transgenic plants of the subject invention.

#### Example 8—WHISKERS-Mediated Transformation of Corn Using Imazethapyr Selection

##### 8.1—Cloning of AAD-12 (v1).

The AAD-12 (v1) gene was cut out of the intermediate vector pDAB3283 as an Nco1/Sac1 fragment. This was ligated directionally into the similarly cut pDAB3403 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5 $\alpha$  cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. This first intermediate construct (pDAB4100) contains the ZmUbi1:AAD-12 (v1) cassette. This construct was digested with Not1 and Pvu1 to liberate the gene cassette and digest the unwanted backbone. This was ligated to Not1 cut pDAB2212, which contains the AHAS selectable marker driven by the Rice Actin promoter OsAct1. The final construct was designated pDAB4101 or pDAS1863, and contains ZmUbi1/AAD-12 (v1)/ZmPer5::OsAct1/AHAS/LZm-Lip.

##### 8.2—Callus/Suspension Initiation.

To obtain immature embryos for callus culture initiation, F<sub>1</sub> crosses between greenhouse-grown Hi-II parents A and B (Armstrong et al. 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% ethanol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu et al., 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L AgNO<sub>3</sub>, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter et al., 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L  $\alpha$ -naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under

62

dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

##### 8.3—Cryopreservation and Thawing of Suspensions.

Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C. for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C. in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C./minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C. until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 ml H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

##### 8.4—Stable Transformation

Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L myo-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker shaken at 125 RPM in the dark for 30-35 minutes at 28° C., and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume 8.1 ml of GN6 S/M liquid medium to ~405 mg of pre-autoclaved, sterile silicon carbide whiskers (Advanced Composite Materials, Inc.).

After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was



US 10,167,483 B2

63

drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was then added to the bottle, to which 170 µg DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker at 125 RPM for 2 hours at 28° C. before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

Approximately 2 ml of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60×20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C. in a dark box.

After 1 week, filter papers were transferred to 60×20 mm plates of GN6 (3P) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 3 µM imazethapyr from Pursuit® DG, 2.5 g/L Gelrite, pH 5.8). Plates were placed in boxes and cultured for an additional week.

Two weeks post-transformation, the tissue was embedded by scraping all cells on the plate into 3.0 ml of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C.) containing 3 µM imazethapyr from Pursuit® DG. The tissue was broken up and the 3 ml of agarose and tissue were evenly poured onto the surface of a 100×15 mm plate of GN6 (3P). This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured until putative isolates appeared.

8.4.1—Protocol for Isolate Recovery and Regeneration.

Putatively transformed events were isolated off the Pursuit®-containing embedded plates approximately 9 weeks post-transformation by transferring to fresh selection medium of the same concentration in 60×20 mm plates. If sustained growth was evident after approximately 2-3 weeks, the event was deemed to be resistant and was submitted for molecular analysis.

Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (3P), containing 3 µM imazethapyr from Pursuit® DG, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light (13 µEm<sup>-2</sup>s<sup>-1</sup>) for one week, then higher light (40 µEm<sup>-2</sup>s<sup>-1</sup>) for another week, before being transferred to regeneration medium, 36 (3P), which was identical to 28 (3P) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150×25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L myo-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

From 4 experiments, full plantlets, comprised of a shoot and root, were formed in vitro on the embedded selection plates under dark conditions without undergoing a traditional callus phase. Leaf tissue from nine of these “early regenerators” were submitted for coding region PCR and Plant Transcription Unit (PTU) PCR for the AAD-12 gene and gene cassette, respectively. All had an intact AAD-12 coding region, while 3 did not have a full-length PTU (Table 15). These “early regenerators” were identified as 4101 events to differentiate them from the traditionally-derived events, which were identified as “1283” events. Plants from

64

19 additional events, obtained via standard selection and regeneration, were sent to the greenhouse, grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T<sub>1</sub> seed. Some of the events appear to be clones of one another due to similar banding patterns following Southern blot, so only 14 unique events were represented. To plants from events were tolerant 70 g/ha imazethapyr. Invader analysis (AHAS gene) indicated insertion complexity ranging from 1 to >10 copies. Thirteen events contained the complete coding region for AAD-12; however, further analysis indicated the complete plant transformation unit had not been incorporated for nine events. None of the compromised 1863 events were advanced beyond the T1 stage and further characterization utilized the 4101 events.

8.5—Molecular Analysis: Maize Materials and Methods.

8.5.1—Tissue Harvesting DNA Isolation and Quantification.

Fresh tissue is placed into tubes and lyophilized at 4° C. for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/µl.

8.5.2—Invader Assay Analysis.

The DNA samples are diluted to 20 ng/µl then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and MgCl<sub>2</sub> (Third Wave Technologies). An aliquot of 7.5 µl is placed in each well of the Invader assay plate followed by an aliquot of 7.5 µl of controls, standards, and 20 ng/µl diluted unknown samples. Each well is overlaid with 15 µl of mineral oil (Sigma). The plates are then incubated at 63° C. for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with Southern blot analysis is used to identify the estimated copy of the unknown events.

8.5.3—Polymerase Chain Reaction.

A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-12 (v1) PTU are Forward-GAACAGTTAGACATG-GTCTAAAGG (SEQ ID NO:8) and Reverse-GCTG-CAACACTGATAAATGCCAACTGG (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 63° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes.

Primers for AAD-12 (v1) Coding Region PCR are Forward-ATGGCTCAGACCACTCTCCAAA (SEQ ID NO:10) and Reverse-AGCTGCATCCATGCCAGGGA (SEQ ID NO: 11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

8.5.4—Southern Blot Analysis.

Southern blot analysis is performed with genomic DNA obtained from Qiagen DNeasy kit. A total of 2 µg of genomic

US 10,167,483 B2

65

leaf DNA or 10 µg of genomic callus DNA is subjected to an overnight digestion using BSM I and SWA I restriction enzymes to obtain PTU data.

After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85% agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INY00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extrac-

66

Plants were sprayed with either a lethal dose of imazethapyr (70 g ae/ha) or a rate of 2,4-D DMA salt capable of significant injury to untransformed corn lines (2240 g ae/ha). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

Several individuals were safened from the herbicides to which the respective genes were to provide resistance. The individual clone '001' from event "001" (a.k.a., 4101(0)-001-001), however, did incur minor injury but recovered by 14 DAT. Three of the four events were moved forward and individuals were crossed with 5XH751 and taken to the next generation. Each herbicide tolerant plant was positive for the presence of the AAD-12 coding region (PCR assay) or the presence of the AHAS gene (Invader assay) for 2,4-D and imazethapyr-tolerant plants, respectively. AAD-12 protein was detected in all 2,4-D tolerant T<sub>0</sub> plants events containing an intact coding region. The copy number of the transgene(s) (AHAS, and by inference AAD-12) varied significantly from 1 to 15 copies. Individual T<sub>0</sub> plants were grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T<sub>1</sub> seed.

TABLE 15

Characterization of T <sub>0</sub> corn plants transformed with AAD-12.						
Event	Spray Treatment	% Injury (14 DAT)	AAD-12 ELISA (ppm TSP)	AAD12 PCR (Coding PCR Region) (PTU)	AHAD Copy # (Invader)	
4101(0)003.001	2240 g ae/ha 2,4-D	0	146.9	+	+	1
4101(0)003.003	2240 g ae/ha 2,4-D	0	153.5	+	+	1
4101(0)005.001	2240 g ae/ha 2,4-D	0	539.7	+	+	9
4101(0)005.0012	0 g ae/ha 2,4-D	0	562.9	+	+	7
4101(0)001.001	70 g ae/ha imazethapyr	5	170.7	+	+	6
4101(0)002.001	0 g ae/ha imazethapyr	0	105.6	+	-	2
4101(0)002.002	70 g ae/ha imazethapyr	0	105.3	+	-	2
4101(0)003.002	70 g ae/ha imazethapyr	0	0	+	band smaller than expected	15

tion procedure. The membrane is then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

#### 8.6—Postemergence Herbicide Tolerance in AAD-12 Transformed T<sub>0</sub> Corn.

Four T<sub>0</sub> events were allowed to acclimate in the greenhouse and were grown until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C. under 16 hour light:8 hour dark conditions in the greenhouse. Plants were then treated with commercial formulations of either Pursuit® (imazethapyr) or 2,4-D Amine 4. Pursuit® was sprayed to demonstrate the function of the selectable marker gene present within the events tested. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height.

#### 8.7—Verification of High 2,4-D Tolerance in T<sub>1</sub> Corn.

T<sub>1</sub> AAD-12 (v1) seed were planted into 3-inch pots containing Metro Mix media and at 2 leaf stage were sprayed with 70 g ae/ha imazethapyr to eliminate nulls. Surviving plants were transplanted to 1-gallon pots containing Metro Mix media and placed in the same growth conditions as before. At V3-V4 stage the plants were sprayed in the track sprayer set to 187 L/ha at either 560 or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to 5XH751×Hi II control plants. A grading scale of 0-10 (no injury to extreme auxin injury) was developed to distinguish brace root injury. Brace Root grades were taken on 14DAT to show 2,4-D tolerance. 2,4-D causes brace root malformation, and is a consistent indicator of auxinic herbicide injury in corn. Brace root data (as seen in the table below) demonstrates that 2 of the 3 events tested were robustly tolerant to 2240 g ae/ha 2,4-D DMA. Event "pDAB4101(0)001.001" was apparently unstable; however, the other two events were robustly tolerant to 2,4-D and 2,4-D+imazethapyr or 2,-4D+glyphosate (see Table 16).

US 10,167,483 B2

67

TABLE 16

Brace Root injury of AAD-12 (v1) transformed T <sub>1</sub> plants and Untransformed control corn plants. A scale of 0-10, 10 being the highest, was used for grading the 2,4-D DMA injury. Results are a visual average of four replications per treatment.				
Herbicide	Untransformed Control	AAD-12 (v1) pDAB4101(0)003.003	AAD-12 (v1) pDAB4101(0)001.001	AAD-12 (v1) pDAB4101(0)005.001
Average Brace Root Injury (0-10 Scale)				
0 g ae/ha 2,4-D DMA	0	0	0	0
2240 g ae/ha 2,4-D DMA	9	1	8	0

68

## 8.8—AAD-12 (v1) Heritability in Corn.

A progeny test was also conducted on seven AAD-12 (v1) T<sub>1</sub> families that had been crossed with 5XH751. The seeds 20 were planted in three-inch pots as described above. At the 3 leaf stage all plants were sprayed with 70 g ae/ha

genes to provide an increased spectrum of herbicides that may be applied safely to corn. Likewise imidazolinone+2, 4-D+glyphosate tolerance was observed in F<sub>1</sub> plants and showed no negative phenotype by the molecular or breeding stack combinations of these multiple transgenes.

TABLE 17

Data demonstrating increase herbicide tolerance spectrum resulting from an F <sub>1</sub> stack of AAD-12 (v1) and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF).				
Herbicide	Untransformed Control	2P782 (Roundup Ready Control)	AAD-12 (v1) pDAB4101(0)003.R003.AF	AAD-12 (v1) pDAB4101(0)005.R001.AF
Average % Injury 16DAT				
0 g ae/ha 2,4-D DMA	0	0	0	0
1120 g ae/ha 2,4-D DMA	21	19	0	0
1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr	100	100	5	1
01120 g ae/ha 2,4-D DMA + 1680 g ae/ha glyphosate	100	71	2	5

imazethapyr in the track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Four out of the six lines tested segregated as a single locus, dominant Mendelian trait (1R:1S) as determined by Chi square analysis. Surviving plants were subsequently sprayed with 2,4-D and all plants were deemed tolerant to 2,4-D 45 (rates  $\geq$  560 g ae/ha). AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species when reciprocally crossed to a commercial hybrid.

## 8.9—Stacking of AAD-12 (v1) to Increase Herbicide Spectrum

AAD-12 (v1) (pDAB4101) and elite Roundup Ready inbred (BE1146RR) were reciprocally crossed and F<sub>1</sub> seed was collected. The seed from two F<sub>1</sub> lines were planted and treated with 70 g ae/ha imazethapyr at the V2 stage to eliminate nulls. To the surviving plants, reps were separated and either treated with 1120 g ae/ha 2,4-D DMA+70 g ae/ha imazethapyr (to confirm presence of AHAS gene) or 1120 g 60 ae/ha 2,4-D DMA+1680 g ae/ha glyphosate (to confirm the presence of the Round Up Ready gene) in a track sprayer calibrated to 187 L/ha. Plants were graded 3 and 16 DAT. Spray data showed that AAD-12 (v1) can be conventionally 65 stacked with a glyphosate tolerance gene (such as the Roundup CP4-EPSPS gene) or other herbicide tolerance

## 8.10—Field Tolerance of DDAB4101 Transformed Corn Plants to 2,4-D, Triclopyr and Fluroxypyr Herbicides.

Field level tolerance trials were conducted on two AAD-12 (v1) pDAB4101 events (4101(0)003.R.003.AF and 4101 (0)005.R001.AF) and one Roundup Ready (RR) control hybrid (2P782) at Fowler, Ind. and Wayside, Miss. Seeds were planted with cone planter on 40-inch row spacing at Wayside and 30 inch spacing at Fowler. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. The AAD-12 (v1) events contained the AHAS gene as a selectable marker. The F<sub>2</sub> corn events were segregating so the AAD-12 (v1) plants were treated with imazethapyr at 70 g ae/ha to remove the null plants. Herbicide treatments were applied when corn reached the V6 stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after treatment. Brace root injury ratings were taken at 28DAT on a scale of 0-10 with 0-1 being slight brace root fusing, 1-3 being moderate brace root swelling/ wandering and root proliferation, 3-5 being moderate brace root fusing, 5-9 severe brace root fusing and malformation and 10 being total inhibition of brace roots.

AAD-12 (v1) event response to 2,4-D, triclopyr, and fluroxypyr at 14 days after treatment are shown in Table 18.

US 10,167,483 B2

69

Crop injury was most severe at 14 DAT. The RR control corn (2P782) was severely injured (44% at 14 DAT) by 2,4-D at 4480 g ae/ha, which is 8 times (8×) the normal field use rate. The AAD-12 (v1) events all demonstrated excellent tolerance to 2,4-D at 14 DAT with 0% injury at the 1, 2 and 4× rates, respectively. The control corn (2P782) was severely injured (31% at 14 DAT) by the 2× rate of triclopyr (840 g ae/ha). AAD-12 (v1) events demonstrated tolerance at 2× rates of triclopyr with an average of 3% injury at 14 DAT across the two events. Fluroxypyr at 280 g ae/ha caused 11% visual injury to the wild-type corn at 14 DAT. AAD-12 (v1) events demonstrated increased tolerance with an average of 8% injury at 5 DAT.

Applications of auxinic herbicides to corn in the V6 growth stage can cause malformation of the brace roots. Table 18 shows the severity of the brace root injury caused by 2,4-D, triclopyr, and fluroxypyr. Triclopyr at 840 g ae/ha caused the most severe brace root fusing and malformation resulting in an average brace root injury score of 7 in the 2P782 control-type corn. Both AAD-12 (v1) corn events showed no brace root injury from the triclopyr treatment. Brace root injury in 2P782 corn increased with increasing rates of 2,4-D. At 4480 g ae/ha of 2,4-D, the AAD-12 events showed no brace root injury; whereas, severe brace root fusing and malformation was seen in the 2P782 hybrid. Fluroxypyr caused only moderate brace root swelling and wandering in the wild-type corn with the AAD-12 (v1) events showing no brace root injury.

This data clearly shows that AAD-12(v1) conveys high level tolerance in corn to 2,4-D, triclopyr and fluroxypyr at rates far exceeding those commercially used and that cause non-AAD-12 (v1) corn severe visual and brace root injury.

TABLE 18

Visual Injury of AAD-12 events and wild-type corn following foliar applications of 2,4-D, triclopyr and fluroxypyr under field conditions.				
% Visual Injury 14 DAT				
Treatment	Rate (g ae/ha)	AAD-12 4101(0)003.- R.003.AF	AAD-12 4101(0)005.- 001.AF	2P782 control
Untreated	0	0	0	0
2,4-D	1120	0	0	9
2,4-D	2240	0	1	20
2,4-D	4480	0	1	34
Fluroxypyr	280	1	5	11
Triclopyr	840	3	4	31
Dicamba	840	8	8	11

TABLE 19

Brace root injury ratings for AAD-12 and wild-type corn plants in response to 2,4-D, triclopyr and fluroxypyr under field conditions.				
Brace Root Injury Rating (0-10 scale) 28 DAT				
Treatment	Rate (g ae/ha)	AAD-12 event 4101(0)003.- R.003.AF	AAD-12 event 4101(0)005.- 001.AF	Wild- type NK603
Untreated	0	0	0	0
2,4-D	1120	0	0	3
2,4-D	2240	0	0	5
2,4-D	4480	0	0	6
Fluroxypyr	280	0	0	2

70

TABLE 19-continued

Brace root injury ratings for AAD-12 and wild-type corn plants in response to 2,4-D, triclopyr and fluroxypyr under field conditions.				
Brace Root Injury Rating (0-10 scale) 28 DAT				
Treatment	Rate (g ae/ha)	AAD-12 event 4101(0)003.- R.003.AF	AAD-12 event 4101(0)005.- 001.AF	Wild- type NK603
Triclopyr	840	0	0	7
Dicamba	840	1	1	1

#### Example 9—Protein Detection from Transformed Plants Via Antibody

##### 9.1—Extracting AAD-12 (v1) from Plant Leaves.

Approximately 50 to 100 mg of leaf tissue was cut into small pieces (or 4 single-hole-punched leaf discs) and put into 2-ml cluster tubes containing 2 stainless steel BB beads (4.5 mm; Daisy Co., cat. #145462-000). Five hundred microliters of plant extraction buffer (PBS containing 0.05% Tween 20 and 1% Bovine serum albumin) was added to each sample. The tubes were capped and secured in the Geno/Grinder (Model 2000-115, Certiprep, Metuchen, N.J.) and shaken for 6 min with setting at 1× of 500 rpm. Tubes were centrifuged at 5000×g for 10 min and supernatant containing the soluble proteins were analyzed for AAD-12 (v1) using Western Blots and ELISA.

##### 9.2—Enzyme Linked Immuno-Sorbent Assay (ELISA).

The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-12 antibody (0.5 µg/ml) was coated on 96-well micro-titer well and incubated at 4° C. for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 µL standard AAD-12 of known concentrations or plant extracts from different samples were incubated in the wells. For standard curve, purified AAD-12 was diluted 2-fold serially from 52 to 0.813 ng/ml in triplicates. Plant extracts were diluted 5, 10, 20, and 40-fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-12 antibody-HRP conjugate (0.5 ug/ml) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce, Rockford, Ill.), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 µL 0.4N H<sub>2</sub>SO<sub>4</sub>. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-12 (v1) in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

For comparison, each sample was normalized with its fresh weight and percent expression was calculated.

##### 9.3—Western Blotting Analysis.

Plant extracts or AAD-12 standards (5 and 0.5 µg/ml) were incubated with Laemmli sample buffer at 95° C. for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-12 (v1) protein was detected by anti-AAD-12 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham, NJ).



US 10,167,483 B2

71

## Example 10—Tobacco Transformation

Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch et al., 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. KY160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C. and leaves collected sterilely for use in the transformation protocol. Pieces of approximately one square centimeter were sterilely cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB3278, aka pDAS1580, AAD-12 (v1)+PAT), grown overnight in a flask on a shaker set at 250 rpm at 28° C., were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Fla.) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C. in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C. greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C., 16 hour day, 8 hour night, minimum natural+supplemental light=500  $\mu\text{E}/\text{m}^2\text{s}^{-1}$ ).

Prior to propagation, T<sub>0</sub> plants were sampled for DNA analysis to determine the insert copy number. The PAT gene which was molecularly linked to AAD-12 (v1) was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico

72

Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ $\mu\text{L}$ .

The DNA samples were diluted to 9 ng/ $\mu\text{L}$  and then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgCl<sub>2</sub> (Third Wave Technologies). An aliquot of 7.5  $\mu\text{L}$  was placed in each well of the Invader assay plate followed by an aliquot of 7.5  $\mu\text{L}$  of controls, standards, and 20 ng/ $\mu\text{L}$  diluted unknown samples. Each well was overlaid with 15  $\mu\text{L}$  of mineral oil (Sigma). The plates were then incubated at 63° C. for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

All events were also assayed for the presence of the AAD-12 (v1) gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit. Primers for the Plant Transcription Unit (PTU) PCR AAD-12 were (SdpacodF: ATG-GCTCA TGCTGCCCTCAGCC) (SEQ ID NO:12) and (SdpacodR: CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO:13). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 64° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 18 PCR positive events with 1-3 copies of PAT gene (and presumably AAD-12 (v1) since these genes are physically linked) were regenerated and moved to the greenhouse.

10.1 Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T<sub>0</sub> Tobacco

T<sub>0</sub> plants from each of the 19 events were challenged with a wide range of 2,4-D, triclopyr, or fluroxypyr sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 140, 560, or 2240 g ae/ha to representative clones from each event mixed in deionized water. Fluroxypyr was likewise applied at 35, 140, or 560 g ae/ha. Triclopyr was applied at 70, 280, or 1120 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 560 g ae/ha 2,4-D or less. Some events were uninjured at 2,4-D applied at 2240 g ae/ha (equivalent to 4 $\times$  field rate). On the whole, AAD-12 (v1) events were more sensitive to fluroxypyr, followed by triclopyr, and least affected by 2,4-D. The quality of the events with respect to magnitude of resistance was discerned using T<sub>0</sub> plant responses to 560 g ae/ha fluroxypyr. Events were categorized into "low" (>40% injury 14 DAT), "medium" (20-40% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable."

US 10,167,483 B2

73

TABLE 20

Tobacco T <sub>0</sub> events transformed with pDAS1580 (AAD-12 (v1) + PAT)						
# Tube	Plant ID	Copy # PAT	PTU PCR AAD-12	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance <sup>@</sup>
1	1580[1]-001	6	+			Not tested
2	1580[1]-002	8	+			Not tested
3	1580[1]-003	10	+			Not tested
4	1580[1]-004	1	+		*	High
5	1580[1]-005	2	+	*		Variable
6	1580[1]-006	6	+			Not tested
7	1580[1]-007	4	+			Not tested
8	1580[1]-008	3	+			Variable
9	1580[1]-009	4	+			Not tested
10	1580[1]-010	8	+			Not tested
11	1580[1]-011	3	+			High
12	1580[1]-012	12	+			Not tested
13	1580[1]-013	13	+			Not tested
14	1580[1]-014	4	+			Not tested
15	1580[1]-015	2	+	*		High
16	1580[1]-016	1 ?	+	*	*	High
17	1580[1]-017	3	+			High
18	1580[1]-018	1	+	*	*	Variable
19	1580[1]-019	1	+	*	*	Variable
20	1580[1]-020	1	+	*	*	Not tested
21	1580[1]-021	1	+	*	*	Not tested
22	1580[1]-022	3	+			Variable
23	1580[1]-023	1	+	*	*	Variable
24	1580[1]-024	1	+	*	*	Variable
25	1580[1]-025	5	+			Not tested
26	1580[1]-026	3	+			Variable
27	1580[1]-027	3	+			Low
28	1580[1]-028	4	+			Not tested
29	1580[1]-029	3	+			Variable
30	1580[1]-030	1	+	*	*	High
31	1580[1]-031	1	+	*	*	High
32	1580[1]-032	2	+	*		High

<sup>@</sup>Distinguishing herbicide tolerance performance of events required assessment of relative tolerance when treated with 560 g ae/ha fluroxypyr where tolerance was variable across events.

### 10.2 Verification of High 2,4-D Tolerance in T<sub>1</sub> Tobacco.

Two to four T<sub>0</sub> individuals surviving high rates of 2,4-D and fluroxypyr were saved from each event and allowed to self fertilize in the greenhouse to give rise to T<sub>1</sub> seed. The T<sub>1</sub> seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 7.4), followed by selective removal of untransformed nulls in this segregating population with 560 g ai/ha glufosinate (PAT gene selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided high levels of resistance to 2,4-D in the T<sub>0</sub> generation. Improved consistency of response is anticipated in T<sub>1</sub> plants not having come directly from tissue culture. These plants were compared against wildtype KY160 tobacco. All plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed from a range of 140-2240 g ae/ha 2,4-D dimethylamine salt (DMA), 70-1120 g ae/ha triclopyr or 35-560 g ae/ha fluroxypyr. All applications were formulated in water. Each treatment was replicated 2-4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T<sub>1</sub> generation is segregating, so some variable response is expected due to difference in zygosity.

No injury was observed at 4× field rate (2240 g ae/ha) for 2,4-D or below. Some injury was observed with triclopyr treatments in one event line, but the greatest injury was observed with fluroxypyr. The fluroxypyr injury was short-

74

lived and new growth on one event was nearly indistinguishable from the untreated control by 14 DAT (Table 21). It is important to note that untransformed tobacco is exceedingly sensitive to fluroxypyr. These results indicated commercial level 2,4-D tolerance can be provided by AAD-12 (v1), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to the pyridyloxyacetic acid herbicides, triclopyr and fluroxypyr. Having the ability to prescribe treatments in an herbicide tolerant crop protected by AAD-12 with various active ingredients having varying spectra of weed control is extremely useful to growers.

TABLE 21

Assessment of cross tolerance of AAD-12 (v1) T <sub>1</sub> tobacco plants' response to various phenoxy and pyridyloxy auxin herbicides.				
Herbicide	KY160- Wildtype generation) Average	1580(1)-004 (high tolerance in T <sub>0</sub> generation)	1580(1)-018 (high tolerance in T <sub>0</sub> generation)	
		% Injury	of Replicates 14 DAT	
140 g ae/ha 2,4-D DMA	45	0	0	
560 g ae/ha 2,4-D DMA	60	0	0	
2240 g ae/ha 2,4-D DMA	73	0	0	
70 g ae/ha triclopyr	40	0	5	
280 g ae/ha triclopyr	65	0	5	
1120 g ae/ha triclopyr	80	0	8	
35 g ae/ha fluroxypyr	85	0	8	
140 g ae/ha fluroxypyr	93	0	10	
560 g ae/ha fluroxypyr	100	3	18	

### 10.3 AAD-12 (v1) Heritability in Tobacco

A 100 plant progeny test was also conducted on seven T<sub>1</sub> lines of AAD-12 (v1) lines. The seeds were stratified, sown, and transplanted with respect to the procedure above with the exception that null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Five out of the seven lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species.

### 10.4—Field Tolerance of pDAS1580 Tobacco Plants to 2,4-D, Dichloprop, Triclopyr and Fluroxypyr Herbicides.

Field level tolerance trials were conducted on three AAD-12 (v1) lines (events pDAS1580-[1]-018.001, pDAS1580-[1]-004.001 and pDAS1580-[1]-020.016) and one wild-type line (KY160) at field stations in Indiana and Mississippi. Tobacco transplants were grown in the greenhouse by planting T<sub>1</sub> seed in 72 well transplant flats (Hummert International) containing Metro 360 media according to growing conditions indicated above. The null plants were selectively removed by Liberty selection as previously described. The transplant plants were transported to the field stations and planted at either 14 or 24 inches apart using industrial vegetable planters. Drip irrigation at the Mississippi site and overhead irrigation at the Indiana site were used to keep plants growing vigorously.

The experimental design was a split plot design with 4 replications. The main plot was herbicide treatment and the sub-plot was tobacco line. The herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and



US 10,167,483 B2

75

4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Plots were one row by 25-30 ft. Herbicide treatments were applied 3-4 weeks after transplanting using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual rating of injury, growth inhibition, and epinasty were taken at 7, 14 and 21 days after treatment.

AAD-12 (v1) event response to 2,4-D, triclopyr, and fluroxypyr are shown in Table 22. The non-transformed tobacco line was severely injured (63% at 14 DAT) by 2,4-D at 560 g ae/ha which is considered the 1× field application rate. The AAD-12 (v1) lines all demonstrated excellent tolerance to 2,4-D at 14 DAT with average injury of 1, 4, and 4% injury observed at the 2, 4 and 8× rates, respectively. The non-transformed tobacco line was severely injured (53% at 14 DAT) by the 2× rate of triclopyr (840 g ae/ha); whereas, AAD-12 (v1) lines demonstrated tolerance with an average of 5% injury at 14 DAT across the three lines. Fluroxypyr at 280 g ae/ha caused severe injury (99%) to the non-transformed line at 14 DAT. AAD-12 (v1) lines demonstrated increased tolerance with an average of 11% injury at 14 DAT.

These results indicate that AAD-12 (v1) transformed event lines displayed a high level of tolerance to 2,4-D, triclopyr and fluroxypyr at multiples of commercial use rates that were lethal or caused severe epinastic malformations to non-transformed tobacco under representative field conditions.

TABLE 22

AAD-12 (v1) tobacco plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.					
		Average % Injury across locations at 14 DAT			
Herbicide Treatment		Wild	pDAS1580-	pDAS1580-	pDAS1580-
Active Ingredient	Rate	type	[1]-004.001	[1]-020.016	[1]-018.001
2,4-D	280 GM AE/HA	48	0	0	0
2,4-D	560 GM AE/HA	63	0	0	2
2,4-D	1120 GM AE/HA	78	1	1	2
2,4-D	2240 GM AE/HA	87	4	4	4
2,4-D	4480 GM AE/HA	92	4	4	4
Triclopyr	840 GM AE/HA	53	5	5	4
Fluroxypyr	280 GM AE/HA	99	11	11	12

#### 10.5 AAD-12 (v1) Protection Against Elevated 2,4-D Rates

Results showing AAD-12 (v1) protection against elevated rates of 2,4-D DMA in the greenhouse are shown in Table 23. T<sub>1</sub> AAD-12 (v1) plants from an event segregating 3R:1S when selected with 560 g ai/ha Liberty using the same protocol as previously described. T<sub>1</sub> AAD-1 (v3) seed was also planted for transformed tobacco controls (see PCT/US2005/014737). Untransformed KY160 was served as the sensitive control. Plants were sprayed using a track sprayer set to 187 L/ha at 140, 560, 2240, 8960, and 35840 g ae/ha 2,4-D DMA and rated 3 and 14 DAT.

AAD-12 (v1) and AAD-1 (v3) both effectively protected tobacco against 2,4-D injury at doses up to 4× commercial use rates. AAD-12 (v1), however, clearly demonstrated a marked advantage over AAD-1 (v3) by protecting up to 64× the standard field rates.

76

TABLE 23

Results demonstrating protection provided by AAD-12 (v1) and AAD-1 (v3) against elevated rates of 2,4-D.			
Treatment	KY160 control	AAD-1 (v3)	AAD-12 (v1)
Average % injury 14 DAT			
2240 g ae/ha 2,4-D	95	4	0
8960 g ae/ha 2,4-D	99	9	0
35840 g ae/ha 2,4-D	100	32	4

#### 10.6 Stacking of AAD-12 to Increase Herbicide Spectrum

Homozygous AAD-12 (v1) (pDAS1580) and AAD-1 (v3) (pDAB721) plants (see PCT/US2005/014737 for the latter) were both reciprocally crossed and F<sub>1</sub> seed was collected. The F<sub>1</sub> seed from two reciprocal crosses of each gene were stratified and treated 4 reps of each cross were treated under the same spray regime as used for the other testing with one of the following treatments: 70, 140, 280 g ae/ha fluroxypyr (selective for the AAD-12 (v1) gene); 280, 560, 1120 g ae/ha R-dichloroprop (selective for the AAD-1 (v3) gene); or 560, 1120, 2240 g ae/ha 2,4-D DMA (to confirm 2,4-D tolerance). Homozygous T<sub>2</sub> plants of each gene were also planted for use as controls. Plants were graded at 3 and 14 DAT. Spray results are shown in Table 24.

The results confirm that AAD-12 (v1) can be successfully stacked with AAD-1 (v3), thus increasing the spectrum

herbicides that may be applied to the crop of interest (phenoxyacetic acids+phenoxypropionic acids vs phenoxyacetic acids+pyridyloxyacetic acids for AAD-1 and AAD-12, respectively). The complementary nature of herbicide cross resistance patterns allows convenient use of these two genes as complementary and stackable field-selectable markers. In crops where tolerance with a single gene may be marginal, one skilled in the art recognizes that one can increase tolerance by stacking a second tolerance gene for the same herbicide. Such can be done using the same gene with the same or different promoters; however, as observed here, stacking and tracking two complementary traits can be facilitated by the distinguishing cross protection to phenoxypropionic acids [from AAD-1 (v3)] or pyridyloxyacetic acids [AAD-12 (v1)]

US 10,167,483 B2

77

78

TABLE 24

Comparison of auxinic herbicide cross tolerance of AAD-12 (v1) (pDAS1580) and AAD-1 (v3) (pDAB721) T <sub>2</sub> plants compared to AAD-12 × AAD-1 F <sub>1</sub> cross and to wildtype.				
Treatment	KY160 wildtype control	AAD-12 (v1) (pDAS1580)	AAD-1(v3) (pDAB721)	AAD-12 (v1) × AAD (v3) F <sub>1</sub>
		Average % injury 14 DAT		
560 g ae/ha 2,4-D	63	0	0	0
1120 g ae/ha 2,4-D	80	0	4	0
2240 g ae/ha 2,4-D	90	0	9	0
280 g ae/ha R-dichlorprop	25	15	0	0
560 g ae/ha R-dichlorprop	60	50	0	0
1120 g ae/ha R-dichlorprop	80	70	3	0
70 g ae/ha fluroxypyr	40	0	40	0
140 g ae/ha fluroxypyr	65	0	60	0
280 g ae/ha fluroxypyr	75	3	75	3

## Example 11—Soybean Transformation

Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgett et al., 1995), amino acid modification (Falco et al., 1995), and insect resistance (Parrott et al., 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe et al., 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchey et al., 1988) or zygotic embryos (Chee et al., 1989) have been reported.

Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee et al., 1989; McCabe et al., 1988), cotyledon (Hinchey et al., 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh et al., 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the AAD-12 (v1) gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using AAD-12 (v1).

## 11.1 Gateway Cloning of Binary Constructs

The AAD-12 (v1) coding sequence was cloned into five different Gateway Donor vectors containing different plant promoters. The resulting AAD-12 (v1) plant expression cassettes were subsequently cloned into a Gateway Destination Binary vector via the LR Clonase reaction (Invitrogen Corporation, Carlsbad Ca, Cat #11791-019).

An NcoI-SacI fragment containing the AAD-12 (v1) coding sequence was digested from DASPICO12 and

ligated into corresponding NcoI-SacI restriction sites within the following Gateway Donor vectors: pDAB3912 (attL1//CsVMV promoter//AtuORF23 3'UTR//attL2); pDAB3916 (attL1//AtUbi10 promoter//AtuORF23 3'UTR//attL2); pDAB4458 (attL1//AtUbi3 promoter//AtuORF23 3'UTR//attL2); pDAB4459 (attL1//ZmUbi1 promoter//AtuORF23 3'UTR//attL2); and pDAB4460 (attL1//AtAct2 promoter//AtuORF23 3'UTR//attL2). The resulting constructs containing the following plant expression cassettes were designated: pDAB4463 (attL1//CsVMV promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4467 (attL1//AtUbi10 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4471 (attL1//AtUbi3 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4475 (attL1//ZmUbi1 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); and pDAB4479 (attL1//AtAct2 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2). These constructs were confirmed via restriction enzyme digestion and sequencing.

The plant expression cassettes were recombined into the Gateway Destination Binary vector pDAB4484 (RB7 MARv3//attR1-ccdB-chloramphenicol resistance-attR2//CsVMV promoter//PATv6//AtuORF1 3'UTR) via the Gateway LR Clonase reaction. Gateway Technology uses lambda phage-based site-specific recombination instead of restriction endonuclease and ligase to insert a gene of interest into an expression vector. Invitrogen Corporation, Gateway Technology: A Universal Technology to Clone DNA Sequences for Functional Analysis and Expression in multiple Systems, Technical Manual, Catalog #'s 12535-019 and 12535-027, Gateway Technology Version E, Sep. 22, 2003, #25-022. The DNA recombination sequences (attL, and attR,) and the LR Clonase enzyme mixture allows any DNA fragment flanked by a recombination site to be transferred into any vector containing a corresponding site. The attL1 site of the donor vector corresponds with attR1 of the binary vector. Likewise, the attL2 site of the donor vector corresponds with attR2 of the binary vector. Using the Gateway Technology the plant expression cassette (from the donor vector) which is flanked by the attL sites can be recombined into the attR sites of the binary vector. The resulting constructs containing the following plant expression cassettes were labeled as: pDAB4464 (RB7 MARv3//CsVMV promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4468 (RB7 MARv3//AtUbi10 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4472 (RB7 MARv3//AtUbi3 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//

US 10,167,483 B2

79

PATv6//AtuORF1 3'UTR); pDAB4476 (RB7 MARv3//ZmUbi1 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); and pDAB4480 (RB7 MARv3//AtAct2 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR) (see Table 8). These constructs were confirmed via restriction enzyme digestion and sequencing.

#### 11.2 Transformation Method 1: Cotyledonary Node Transformation of Soybean Mediated by *Agrobacterium tumefaciens*.

The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee et al., 1988) and shoot multiplication from apical meristems (McCabe et al., 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee et al., 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente et al., 2000; Olhoft et al., 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe et al., 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell et al., 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This de novo meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee et al., 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang et al., 1999; Zeng et al., 2004).

##### 11.2.1—Plant Transformation Production of AAD-12 (v1) Tolerant Phenotypes.

Seed derived explants of "Maverick" and the *Agrobacterium* mediated cot-node transformation protocol was used to produce AAD-12 (v1) transgenic plants.

##### 11.2.2—*Agrobacterium* Preparation and Inoculation

*Agrobacterium* strain EHA101 (Hood et al. 1986), carrying each of five binary pDAB vectors (Table 8) was used to initiate transformation. Each binary vector contains the AAD-12 (v1) gene and a plant-selectable gene (PAT) cassette within the T-DNA region. Each gene is driven by the promoters listed in Table 8 and these plasmids were mobilized into the EHA101 strain of *Agrobacterium* by electroporation. The selected colonies were then analyzed for the integration of genes before the *Agrobacterium* treatment of the soybean explants. Maverick seeds were used in all transformation experiments and the seeds were obtained from University of Missouri, Columbia, Mo.

*Agrobacterium*-mediated transformation of soybean (*Glycine max*) using the PAT gene as a selectable marker coupled with the herbicide glufosinate as a selective agent was carried out followed a modified procedure of Zeng et al. (2004). The seeds were germinated on B5 basal medium

80

(Gamborg et al. 1968) solidified with 3 g/L Phytigel (Sigma-Aldrich, St. Louis, Mo.); added 1-cysteine to the co-cultivation medium at 400 mg/L and co-cultivation lasted 5 days (Olhoft and Somers 2001); shoot initiation, shoot elongation, and rooting media were supplemented with 50 mg/L cefotaxime, 50 mg/L timentin, 50 mg/L vancomycin, and solidified with 3 g/L Phytigel. Selected shoots were then transferred to the rooting medium. The optimal selection scheme was the use of glufosinate at 8 mg/L across the first and second shoot initiation stages in the medium and 3-4 mg/L during shoot elongation in the medium.

Prior to transferring elongated shoots (3-5 cm) to rooting medium, the excised end of the internodes were dipped in 1 mg/L indole 3-butyric acid for 1-3 min to promote rooting (Khan et al. 1994). The shoots struck roots in 25×100 mm glass culture tubes containing rooting medium and then they were transferred to soil mix for acclimatization of plantlets in Metro-mix 200 (Hummert International, Earth City, Mo.) in open Magenta boxes in Conviron. Glufosinate, the active ingredient of Liberty herbicide (Bayer Crop Science), was used for selection during shoot initiation and elongation. The rooted plantlets were acclimated in open Magenta boxes for several weeks before they were screened and transferred to the greenhouse for further acclimation and establishment.

##### 11.2.3—Assay of Putatively Transformed Plantlets, and Analyses Established T<sub>0</sub> Plants in the Greenhouse.

The terminal leaflets of selected leaves of these plantlets were leaf painted with 50 mg/L of glufosinate twice with a week interval to observe the results to screen for putative transformants. The screened plantlets were then transferred to the greenhouse and after acclimation the leaves were painted with glufosinate again to confirm the tolerance status of these plantlets in the GH and deemed to be putative transformants.

Plants that are transferred to the greenhouse can be assayed for the presence of an active PAT gene further with a non-destructive manner by painting a section of leaf of the T<sub>0</sub> primary transformant, or progeny thereof, with a glufosinate solution [0.05-2% v/v Liberty Herbicide, preferably 0.25-1.0% (v/v), =500-2000 ppm glufosinate, Bayer Crop Science]. Depending on the concentration used, assessment for glufosinate injury can be made 1-7 days after treatment. Plants can also be tested for 2,4-D tolerance in a non-destructive manner by selective application of a 2,4-D solution in water (0.25-1% v/v commercial 2,4-D dimethylamine salt formulation, preferably 0.5% v/v=2280 ppm 2,4-D ae) to the terminal leaflet of the newly expanding trifoliolate one or two, preferably two, nodes below the youngest emerging trifoliolate. This assay allows assessment of 2,4-D sensitive plants 6 hours to several days after application by assessment of leaf flipping or rotation >90 degrees from the plane of the adjacent leaflets. Plants tolerant to 2,4-D will not respond to 2,4-D. T<sub>0</sub> plants will be allowed to self fertilize in the greenhouse to give rise to T<sub>1</sub> seed. T<sub>1</sub> plants (and to the extent enough T<sub>0</sub> plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by AAD-12 (v1) and PAT genes in transgenic soybean. Rates of 2,4-D used on T<sub>0</sub> plants will typically comprise one or two selective rates in the range of 100-1120 g ae/ha using a track sprayer as previously described. T<sub>1</sub> plants will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise, T<sub>0</sub> and T<sub>1</sub> plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Glyphosate resistance (in plants transformed with constructs that contain EPSPS) or another glyphosate tolerance gene can be

## US 10,167,483 B2

## 81

assessed in the T<sub>1</sub> generation by postemergence applications of glyphosate with a dose range from 280-2240 g ae/ha glyphosate. Analysis of protein expression will occur as described in below. Individual T<sub>0</sub> plants were assessed for the presence of the coding region of the gene of interest (AAD-12 (v1) or PAT v6) and copy number. Determination of the inheritance of AAD-12 (v1) will be made using T<sub>1</sub> and T<sub>2</sub> progeny segregation with respect to herbicide tolerance as described in previous examples.

A subset of the initial transformants were assessed in the T<sub>0</sub> generation according to the methods above. Any plant confirmed as having the AAD-12 (v1) coding region, regardless of the promoter driving the gene did not respond to the 2,4-D leaf painting whereas wildtype Maverick soybeans did (Table Sec 11.2.3). PAT-only transformed plants responded the same at wildtype plants to leaf paint applications of 2,4-D

2,4-D was applied to a subset of the plants that were of similar size to the wildtype control plants with either 560 or 1120 g ae 2,4-D. All AAD-12 (v1)-containing plants were clearly resistant to the herbicide application versus the wildtype Maverick soybeans. A slight level of injury (2 DAT) was observed for two AAD-12 (v1) plants, however, injury was temporary and no injury was observed 7 DAT. Wildtype control plants were severely injured 7-14 DAT at 560 g ae/ha 2,4-D and killed at 1120 g ae/ha. These data are consistent with the fact that AAD-12 (v1) can impart high tolerance (>2× field rates) to a sensitive crop like soybeans. The screened plants were then sampled for molecular and biochemical analyses for the confirmation of the AAD12 (v1) genes integration, copy number, and their gene expression levels as described below and reported in Table 25.

## 82

## 11.2.4—Molecular Analyses: Soybean

## 11.2.4.1—Tissue Harvesting DNA Isolation and Quantification.

Fresh tissue is placed into tubes and lyophilized at 4° C. for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μL.

## 11.2.4.2—Polymerase Chain Reaction.

A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-12 (v1) PTU are (Forward-ATAATGCCAGC CTGT-TAAACGCC) (SEQ ID NO:8) and (Reverse-CT-CAAGCATATGAATGACCT CGA) (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 63° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. Primers for Coding Region PCR AAD-12 (v1) are (Forward-ATGGCT-CATGCTGCCCTCAGCC) (SEQ ID NO:10) and (Reverse-CGGGC AGGCCTAACTCCACCAA) (SEQ ID NO: 11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute

TABLE 25

T <sub>0</sub> soybean response to 2,4-D leaf paint and 2,4-D spray application.														
Leaf flip assay NODE 2,4-D @ (18 HAT)														
Construct				LEAF PAINT-	Node	Spray POST over the top with 2,4-D		Stage at appl (#	ELISA]	South-ern Copy	PCR coding	% injury 2	% injury 7	% injury 14
(pDAB#)	Gene	Promoter	Event	ED	N-1	N-2	(g ae/ha)	nodes)	(ng/ML)	Number	region	DAT	DAT	DAT
4464	AAD-12	CsVMV	D-1-14	N-1	0		0	>10	5246.83	2	+	X	X	0
4464	AAD-12	CsVMV	D-2-9	N-2		0	0	>10	204.27	1	+	X	X	0
4468	AAD-12	AtUbi10	D-3-7	N-2		0	0	>10	4.65	1	+	0	0	0
4468	AAD-12	AtUbi10	D-4-11B	N-2		0	0	8	1452.84	2	+	0	0	0
4468	AAD-12	AtUbi10	D-4-16	N-2		0	0	>10	653.21	2	+	X	X	0
4480	AAD-12	AtAct2	D-9-1	N-2		0	0	>10	248.33	3 or 4	+	X	X	0
4464	AAD-12	CsVMV	D-2-14	N-2		0	560	7	4917.43	2	+	0	0	0
4468	AAD-12	AtUbi10	D-3-5	N-2		0	560	8	365.75	1	+	0	0	0
4468	AAD-12	AtUbi10	D-3-6	N-1	0		560	5	714.79	3	+	0	0	0
4472	AAD-12	AtUbi3	D-5-2	N-1	0		560	6	0.58	1	+	5	0	0
4468	AAD-12	AtUbi10	D-3-9	N-2		0	1120	6	2657.26	3	+	0	0	0
4468	AAD-12	AtUbi10	D-4-17	N-2		0	1120	7	286.14	5	+	5	0	0
4499	PAT	CsVMV	D-2-3	N-2		1	0	>10	2.36	5	+	X	X	0
Maverick	WT		WT-10	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-2	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-3	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-4	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-5	NT			560	4	ND	ND	ND	50	60	60
Maverick	WT		WT-6	NT			560	4	ND	ND	ND	70	90	80
Maverick	WT		WT-7	NT			560	4	ND	ND	ND	70	80	80
Maverick	WT		WT-10	NT			1120	4	ND	ND	ND	70	90	100
Maverick	WT		WT-8	NT			1120	4	ND	ND	ND	70	95	100
Maverick	WT		WT-9	NT			1120	4	ND	ND	ND	70	95	100

1 = Flip 0 = No Flip  
ND = Not determined



US 10,167,483 B2

83

and 45 seconds followed by 72° C. for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

#### 11.2.4.3—Southern Blot Analysis.

Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 10 µg of genomic DNA is subjected to an overnight digestion to obtain integration data. After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85% agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

#### 11.2.5—Biochemical Analyses: Soybean

##### 11.2.5.1—Tissue Sampling and Extracting AAD-12 (v1) Protein from Soybean Leaves.

Approximately 50 to 100 mg of leaf tissue was sampled from the N-2 leaves that were 2,4-D leaf painted, but after 1 DAT. The terminal N-2 leaflet was removed and either cut into small pieces or 2-single-hole-punched leaf discs (~0.5 cm in diameter) and were frozen on dry ice instantly. Further protein analysis (ELISA and Western analysis) was completed according to methods described in Example 9.

##### 11.2.6—T<sub>1</sub> Progeny Evaluation.

T<sub>0</sub> plants will be allowed to self fertilize to derive T<sub>1</sub> families. Progeny testing (segregation analysis) will be assayed using glufosinate at 560 g ai/ha as the selection agent applied at the V1-V2 growth stage. Surviving plants will be further assayed for 2,4-D tolerance at one or more growth stages from V2-V6. Seed will be produced through self fertilization to allow broader herbicide testing on the transgenic soybean.

AAD-12 (v1) transgenic Maverick soybean plants have been generated through *Agrobacterium*-mediated cot-node transformation system. The T<sub>0</sub> plants obtained tolerated up to 2× levels of 2,4-D field applications and developed fertile seeds. The frequency of fertile transgenic soybean plants was up to 5.9%. The integration of the AAD1-12 (v1) gene into the soybean genome was confirmed by Southern blot analysis. This analysis indicated that most of the transgenic plants contained a low copy number. The plants screened with AAD-12 (v1) antibodies showed positive for ELISA and the appropriate band in Western analysis.

84

#### 11.3 Transformation Method 2: Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue.

Culture of embryogenic soybean callus tissue and subsequent beaming can be accomplished as described in U.S. Pat. No. 6,809,232 (Held et al.) to create transformants using one or more constructs in Table 8.

#### 11.4 Transformation Method 3. Biolistic Bombardment of Soybean

This can be accomplished using mature seed derived embryonic axes meristem (McCabe et al. (1988)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

#### 11.5 Transformation Method 4. Whiskers Mediated Transformation.

Whisker preparation and whisker transformation can anticipated according to methods described previously by Terakawa et al. (2005)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

Maverick seeds were surface-sterilized in 70% ethanol for 1 min followed by immersion in 1% sodium hypochlorite for 20 min. and then rinsed three times in sterile distilled water. The seeds were soaked in distilled water for 18-20 h. The embryonic axes were excised from seeds, and the apical meristems were exposed by removing the primary leaves. The embryonic axes were positioned in the bombardment medium [BM: MS (Murashige and Skoog 1962) basal salts medium, 3% sucrose and 0.8% phytigel Sigma, pH 5.7] with the apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium.

#### 11.6 Transformation Method 5.

Particle bombardment-mediated transformation for embryogenic callus tissue can be optimized for according to previous methods (Khalafalla et al., 2005; El-Shemy et al., 2004, 2006). Regenerated plants can also be assessed according to Example 11.2.

### Example 12—AAD-12 (v1) in Cotton

#### 12.1—Cotton Transformation Protocol.

Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50% commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (Table 26) in Magenta GA-7 vessels and maintained under high light intensity of 40-60 µE/m<sup>2</sup>, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

Cotyledon segments (~5 mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (Table 26) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (Table 26) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (Table 26). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

#### *Agrobacterium* Preparation.

Inoculate 35 ml of Y media (Table 26) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml

US 10,167,483 B2

85

stock)), with one loop of bacteria to grow overnight in the dark at 28° C., while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (Table 26) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-

86

Larger, well-developed embryos are isolated and transferred to DK media (Table 26) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

TABLE 26

Media for Cotton Transformation							
Ingredients in 1 liter	G	M liquid	M	MG	D	DK	Y
LS Salts (5x)	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1 mM)		1 ml	1 ml	1 ml		0.5 ml	
2,4-D (1 mM)		1 ml	1 ml	1 ml			
Agar	8 grams		8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)					1 package	1 package	
MYO-Inositol (100x)					1 ml	10 ml	
Sucrose 3%	30 grams					30 grams	10 grams
NAA							
Carbenicillin (250 mg/ml)				2 ml			
GLA (10 mg/ml)				0.5 ml			
Peptone							10 grams
Yeast Extract							10 grams
NaCl							5 grams

3). Dilute the new suspension using M liquid media to a Klett-meter reading of  $10^8$  colony forming units per ml with a total volume of 40 ml.

After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. In a side-by-side comparison, MG media can be supplemented with dichlorprop (added to the media at a concentration of 0.01 and 0.05 mg/L) to supplement for the degradation of the 2,4-D, since dichlorprop is not a substrate for to the AAD-12 enzyme, however dichlorprop is more active on cotton than 2,4-D. In a separate comparison, segments which were plated on MG media containing no growth regulator compared to standard MG media, showed reduced callusing, but there still is callus growth. Callus is then transferred to CG-media (Table 26), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 26) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color. Cotton can take time to regenerate and form embryos, one of the ways to speed up this process is to stress the tissue. Dessication is a common way to accomplish this, via changes in the microenvironment of the tissue and plate, by using less culture media and/or adopting various modes of plate enclosure (taping versus parafilm).

#### 12.2—Cell Transformation.

7 Several experiments were initiated in which cotyledon segments were treated with *Agrobacterium* containing pDAB724. Over 2000 of the resulting segments were treated using various auxin options for the proliferation of pDAB724 cotton callus, either: 0.1 or 0.5 mg/L R-dichlorprop, standard 2,4-D concentration and no auxin treatment. The callus was selected on glufosinate-ammonium, due to the inclusion of the PAT gene in the construct. Callus line analysis in the form of PCR and Invader will be used to determine if and to be sure the gene was present at the callus stage; then callus lines that are embryogenic will be sent for Western analysis, essentially as described in section 11.2.3. Embryogenic cotton callus was stressed using dessication techniques to improve the quality and quantity of the tissue recovered.

Almost 200 callus events have been screened for intact PTU and expression using Western analysis for the AAD-12 (v1) gene. Below is a subset of the data for some of the cotton callus that has been tested.

TABLE 26.b

Construct	Line Number	AAD-12 PTU	AAD-12 Invader	AAD-12 ng/ml
pDAB724	1	+	+	79.89
pDAB724	2	+	+	17.34
pDAB724	3	+	+	544.80



US 10,167,483 B2

87

TABLE 26.b-continued

Construct	Line Number	AAD-12 PTU	AAD-12 Invader	AAD-12 ng/ml
pDAB724	4	+	+	32.63
pDAB724	5	+	+	82.77
pDAB724	83	+	+	795.50
pDAB724	84	+	+	613.35
pDAB724	85	+	+	1077.75
pDAB724	86	+	+	437.74
pDAB724	87	+	+	286.51
pDAB724	88	+	+	517.59
pDAB724	89	+	+	1250.70

## 12.3—Plant Regeneration.

AAD-12 (v1) cotton lines that have produced plants according to the above protocol will be sent to the greenhouse. To demonstrate the AAD-12 (v1) gene provides resistance to 2,4-D in cotton, both the AAD-12 (v1) cotton plant and wild-type cotton plants will be sprayed with a track sprayer delivering 560 g ae/ha 2,4-D at a spray volume of 187 L/ha. The plants will be evaluated at 3 and 14 days after treatment. Plants surviving a selective rate of 2,4-D will be self pollinated to create T<sub>1</sub> seed or outcrossed with an elite cotton line to produce F<sub>1</sub> seed. The subsequent seed produced will be planted and evaluated for herbicide resistance as previously described. AAD-12 (v1) events can be combined with other desired HT or IR traits as described in experiments 18, 19, 22, and 23.

Example 13—*Agrobacterium* Transformation of Other Crops

In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated transformation of rye, see, e.g., Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, see, e.g., Hinchey et al., 1988. For *Agrobacterium*-mediated transformation of sorghum, see, e.g., Zhao et al., 2000. For *Agrobacterium*-mediated transformation of barley, see, e.g., Tingay et al., 1997. For *Agrobacterium*-mediated transformation of wheat, see, e.g., Cheng et al., 1997. For *Agrobacterium*-mediated transformation of rice, see, e.g., Hiei et al., 1997.

The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform AAD-12 (v1), for example, into these and other plants, including but not limited to Maize (*Zea mays*), Wheat (*Triticum* spp.), Rice (*Oryza* spp. and *Zizania* spp.), Barley (*Hordeum* spp.), Cotton (*Abroma augusta* and *Gossypium* spp.), Soybean (*Glycine max*), Sugar and table beets (*Beta* spp.), Sugar cane (*Arenga pinnata*), Tomato (*Lycopersicon esculentum* and other spp.), *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*), Potato (*Solanum tuberosum*), Sweet potato (*Ipomoea batatas*), Rye (*Secale* spp.), Peppers (*Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Lactuca sativa*, *perennis*, and *pulchella*), Cabbage (*Brassica* spp), Celery (*Apium graveolens*), Eggplant (*Solanum melongena*), Peanut (*Arachis hypogea*), Sorghum (all *Sorghum* species), Alfalfa (*Medicago sativa*), Carrot (*Daucus carota*), Beans (*Phaseolus* spp. and other genera), Oats (*Avena sativa* and *strigosa*), Peas (*Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Helianthus annuus*), Squash (*Cucurbita* spp.), Cucumber (*Cucumis sativa*), Tobacco (*Nicotiana* spp.), *Arabidopsis* (*Arabidopsis thaliana*), Turfgrass (*Lolium*, *Agrostis*, *Poa*,

88

*Cynodon*, and other genera), Clover (*Trifolium*), Vetch (*Vicia*). Such plants, with AAD-12 (v1) genes, for example, are included in the subject invention.

AAD-12 (v1) has the potential to increase the applicability of key auxinic herbicides for in-season use in many deciduous and evergreen timber cropping systems. Triclopyr, 2,4-D, and/or fluroxypyr resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited to: Alder (*Alnus* spp.), ash (*Fraxinus* spp.), aspen and poplar species (*Populus* spp.), beech (*Fagus* spp.), birch (*Betula* spp.), cherry (*Prunus* spp.), eucalyptus (*Eucalyptus* spp.), hickory (*Carya* spp.), maple (*Acer* spp.), oak (*Quercus* spp), and pine (*Pinus* spp). Use of auxin resistance for the selective weed control in ornamental and fruit-bearing species is also within the scope of this invention. Examples could include, but not be limited to, rose (*Rosa* spp.), burning bush (*Euonymus* spp.), petunia (*Petunia* spp), begonia (*Begonia* spp.), rhododendron (*Rhododendron* spp), crabapple or apple (*Malus* spp.), pear (*Pyrus* spp.), peach (*Prunus* spp), and marigolds (*Tagetes* spp.).

## Example 14—Further Evidence of Surprising Results: AAD-12 vs. AAD-2

## 14.1—AAD-2 (v1) Initial Cloning.

Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to tfdA. This gene is referred to herein as AAD-2 (v1) for consistency. Percent identity was determined by first translating both the AAD-2 and tfdA DNA sequences (SEQ ID NO:12 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

The strain of *Bradyrhizobium japonicum* containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C. in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C. for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C. on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward (SEQ ID NO:16): 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse (SEQ ID NO:17): 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(brjap 3' (xhoI) SEQ ID NO:15 of PCT/US2005/014737 (added a Xho I site)].

Fifty microliter reactions were set up as follows: Fail Safe Buffer 25 µl, ea. primer 1 µl (50 ng/µl), gDNA 1 µl (200 ng/µl), H<sub>2</sub>O 21 µl, Taq polymerase 1 µl (2.5 units/µl). Three Fail Safe Buffers—A, B, and C—were used in three separate reactions. PCR was then carried out under the following conditions: 95° C. 3.0 minutes heat denature cycle; 95° C. 1.0 minute, 50° C. 1.0 minute, 72° C. 1.5 minutes, for 30 cycles; followed by a final cycle of 72° C. 5 minutes, using

US 10,167,483 B2

89

the FailSafe PCR System (Epicenter cat. #FS99100). The resulting ~1 kb PCR product was cloned into pCR 2.1 (Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F<sup>+</sup> *E. coli* as the host strain, for verification of nucleotide sequence.

Ten of the resulting white colonies were picked into 3 µl Luria Broth+1000 µg/ml Ampicillin (LB Amp), and grown overnight at 37° C. with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA G 3'] (SEQ ID NO:6) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:7) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation AAD-2 (v1) for internal consistency.

#### 14.2—Completion of AAD-2 (v1) Binary Vector.

The AAD-2 (v1) gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. See PCT/US2005/014737. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:14) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:15) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI AAD-2 (v1) gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: AAD-2 (v1); Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI-NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: AAD-2 (v1); Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter PAT: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HindIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

#### 14.3—Evaluation of Transformed *Arabidopsis*.

Freshly harvested T<sub>1</sub> seed transformed with a plant optimized AAD-12 (v1) or native AAD-2 (v1) gene were planted and selected for resistance to glufosinate as previously described. Plants were then randomly assigned to various rates of 2,4-D (50-3200 g ae/ha). Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.) mixed in 200 mM Tris buffer (pH 9.0) or 200 mM HEPES buffer (pH7.5).

AAD-12 (v1) and AAD-2 (v1) did provide detectable 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T<sub>1</sub> *Arabidopsis* plants. Surprisingly, AAD-2 (v1) and AAD-2 (v2) transformants were far less resistant to 2,4-D

90

than the AAD-12 (v1) gene, both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with AAD-2 (v1) survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (see PCT/US2005/014737). Conversely, AAD-12 (v1) had a population injury average of about 6% when treated with 3,200 g ae/ha 2,4-D (Table 11). Tolerance improved slightly for plant-optimized AAD-2 (v2) versus the native gene; however, comparison of both AAD-12 and AAD-2 plant optimized genes indicates a significant advantage for AAD-12 (v1) in planta.

These results are unexpected given that the in vitro comparison of AAD-2 (v1) (see PCT/US2005/014737) and AAD-12 (v2) indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. AAD-2 (v1) is expressed in individual T<sub>1</sub> plants to varying levels; however, little protection from 2,4-D injury is afforded by this expressed protein. No substantial difference was evident in protein expression level (in planta) for the native and plant optimized AAD-2 genes (see PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of AAD-12 (v1) in planta, and resulting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides, unexpected.

#### Example 15—Preplant Burndown Applications

This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-12 invention.

Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ae/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280-1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ae/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2005 or online at [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)). With AAD-

US 10,167,483 B2

91

12 (v1) transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone+2,4-D or glufosinate+2, 4-D by utilizing products described in federal herbicide labels (CPR, 2005) and uses described in Agrilience Crop Protection Guide (2005), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-12 (v1) gene if stably transformed. Likewise, the unique attributes of AAD-12 allowing degradation of triclopyr and fluroxypyr increase utility by allowing substitution or tank mixes of 70-1120 or 35-560 g ae/ha of triclopyr and fluroxypyr, respectively, to increase spectram and/or increase the ability to control perennial or viney weed species.

Example 16—In-Crop Use of Phenoxy Auxins  
Herbicides in Soybeans, Cotton, and Other Dicot  
Crops Transformed Only with AAD-12 (v1)

AAD-12 (v1) can enable the use of phenoxy auxin herbicides (e.g., 2,4-D and MCPA) and pyridyloxy auxins (triclopyr and fluroxypyr) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560-1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 g ae/ha.

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, and fluroxypyr when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to combine herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy.

A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetamide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

Further benefits could include tolerance to 2,4-D, triclopyr or fluroxypyr required before planting following aryloxyacetic acid auxin herbicide application (see previous

92

example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks that had contained 2,4-D, triclopyr or fluroxypyr. Dicamba (and many other herbicides) can still be used for the subsequent control of AAD-12 (v1)-transformed dicot crop volunteers.

Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other aryloxy auxin herbicide) crop that would be protected by the AAD-12 (v1) gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by AAD-12 (v1) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTCs by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 17—In-Crop Use of Phenoxy Auxin and  
Pyridyloxy Auxin Herbicides in AAD-12 (v1) Only  
Transformed Corn, Rice, and Other Monocot  
Species

In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-12 (v1) would allow the use of highly efficacious phenoxy and pyridyloxy auxins in crops where normally selectivity is not certain. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D,). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing or unacceptable injury risk. AAD-12 (v1)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 g ae/ha.

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, or fluroxypyr. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-12 (v1) HTC combination strategy, whether one rotates crops species or not.

A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would



US 10,167,483 B2

93

recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetamide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

The increased tolerance of corn, rice, and other monocots to the phenoxy or pyridyloxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as “rat-tailing,” crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots. Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

#### Example 18—AAD-12 (v1) Stacked With Glyphosate Tolerance Trait in Any Crop

The vast majority of cotton, canola, corn, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, *petunia*, and begonias; [isb.vt.edu/cfdocs/fieldtests1.cfm](http://isb.vt.edu/cfdocs/fieldtests1.cfm), 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking AAD-12 (v1) with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-12 (v1), monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and a GT trait are stacked in any monocot or dicot crop species:

- a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (e.g., *Commelina* spp, *Ipomoea* spp, etc), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.
- b) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. AAD-12 (v1)+GT stacked

94

traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha). 2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. Triclopyr and fluroxypyr at rates mentioned above would be acceptable components in the treatment regimen. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D, triclopyr, or fluroxypyr.

One skilled in the art of weed control will recognize that use of one or more commercial aryloxy auxin herbicides alone or in combination (sequentially or independently) is enabled by AAD-12 (v1) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manufact.asp](http://cdms.net/manuf/manufact.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

#### Example 19—AAD-12 (v1) Stacked with Glufosinate Tolerance Trait in any Crop

Glufosinate tolerance (PAT or bar) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (e.g., rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and “burning” of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agrilience, 2005).

By stacking AAD-12 (v1) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

- a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.
- i) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis*, *Apocynum cannabinum*, and *Conyza canadensis*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve

## US 10,167,483 B2

95

the robustness of control on annual broadleaf weed species. Triclopyr and fluroxypyr would be acceptable components to consider in the weed control regimen. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

- b) A multiple combination of glufosinate (200-500 g ae/ha)+/-2,4-D (280-1120 g ae/ha)+/-triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of one or more commercial aryloxyacetic auxin herbicides alone or in combination (sequentially or independently) is enabled by AAD-12 (v1) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 20—AAD-12 (v1) Stacked with AHAS Trait in any Crop

Imidazolinone herbicide tolerance (AHAS, et al.) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapyr has been enabled through imidazolinone tolerance traits like AHAS et al. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agrilience, 2005). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2005). By stacking AAD-12 (v1) with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-12 (v1), monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and these auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

96

- a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.

i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis*, *Ambrosia trifida*, *Chenopodium album* (among others, Heap, 2005) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea* spp. can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. See rates above for triclopyr or fluroxypyr.

- b) A multiple combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha)+/-2,4-D (280-1120 g ae/ha)+/-triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by AAD-12 (v1) transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., cdms.net/manuf/manuf.asp), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 21—AAD-12 (v1) in Rice

21.1—Media Description.

Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/L Phytagel (Sigma). Embryogenic calli were cultured in 100x20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25-26° C., and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang et al. 1996).

Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li et al. 1993), but adapted to contain 500 mg/L glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang et al. 1998) with the inclusion of 30 g/L sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/L hygromycin B for 3-4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of

US 10,167,483 B2

97

2 mg/L 6-benzylaminopurine (BAP), 1 mg/L  $\alpha$ -naphthale-neacetic acid (NAA), 5 mg/L abscisic acid (ABA) and 50 mg/L hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50) comprising NB medium without 2,4-D, and supplemented with 3 mg/L BAP, 0.5 mg/L NAA, and 50 mg/L hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/L hygromycin B ( $\frac{1}{2}$ MSH50).

#### 21.2—Tissue Culture Development.

Mature desiccated seeds of *Oryza sativa* L. *japonica* cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing  $\frac{1}{2}$  MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on  $\frac{1}{2}$  MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week, prior to storage.

#### 21.3—Microprojectile Bombardment.

All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50  $\mu$ l water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB4101 (AAD-12 (v1)+AHAS), 20  $\mu$ l spermidine (0.1 M) and 50  $\mu$ l calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60  $\mu$ l cold 100% ethanol and 8-9  $\mu$ l was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang et al. (1996).

#### 21.4—Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed to Rice

Rice plantlets at the 3-5 leaf stage were sprayed with a lethal dose of 0.16% (v/v) solution of Pursuit (to confirm the

98

presence of the AHAS gene) containing 1% Sunit II (v/v) and 1.25% UAN (v/v) using a track sprayer calibrated to 187 L/ha. Rating for sensitivity or resistance was performed at 36 days after treatment (DAT). Ten of the 33 events sent to the greenhouse were robustly tolerant to the Pursuit; others suffered varying levels of herbicide injury. Plants were sampled (according to section 21.7 below) and molecular characterization was performed as previously described in Example 8 that identified seven of these 10 events as containing both the AAD-12 (v1) PTU and the entire AHAS coding region.

#### 21.5—Heritability of AAD-12 (v1) in T<sub>1</sub> Rice

A 100-plant progeny test was conducted on five T<sub>1</sub> lines of AAD-12 (v1) lines that contained both the AAD-12 (v1) PTU and AHAS coding region. The seeds were planted with respect to the procedure above and sprayed with 140 g ae/ha imazethapyr using a track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Two out of the five lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 cosegregated with the AHAS selectable marker as determined by 2,4-D tolerance testing below.

#### 21.6—Verification of High 2,4-D Tolerance in T<sub>1</sub> Rice.

The following T<sub>1</sub> AAD-12 (v1) single segregating locus lines were planted into 3-inch pots containing Metro Mix media: pDAB4101(20)003 and pDAB4101(27)002. At 2-3 leaf stage were sprayed with 140 g ae/ha imazethapyr. Nulls were eliminated and individuals were sprayed at V3-V4 stage in the track sprayer set to 187 L/ha at 1120, 2240 or 4480 g ae/ha 2,4-D DMA (2x, 4x, and 8x typical commercial use rates, respectively). Plants were graded at 7 and 14 DAT and compared to untransformed commercial rice cultivar, 'Lamont,' as negative control plants.

Injury data (Table 27) shows that the AAD-12 (v1)-transformed lines are more tolerant to high rates of 2,4-D DMA than the untransformed controls. The line pDAB4101 (20)003 was more tolerant to high levels of 2,4-D than the line pDAB4101(27)002. The data also demonstrates that tolerance of 2,4-D is stable for at least two generations.

TABLE 27

T <sub>1</sub> AAD-12 (v1) and untransformed control response to varying levels of 2,4-D DMA.			
Herbicide	Lemont Untransformed Control	pDAB4101(20)003	pDAB4101(27)002
	Average % Injury 14 DAT		
1120 g ae/ha 2,4-D DMA	20	10	10
2240 g ae/ha 2,4-D DMA	35	15	30
4480 g ae/ha 2,4-D DMA	50	23	40

#### 21.7—Tissue Harvesting, DNA Isolation and Quantification.

Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ $\mu$ l.



US 10,167,483 B2

99

## 21.8—AAD-12 (v1) Expression.

Sample preparation and analysis conditions were as described previously. All 33 T<sub>0</sub> transgenic rice lines and 1 non-transgenic control were analyzed for AAD-12 expression using ELISA blot. AAD-12 was detected in the clones of 20 lines, but not in line Taipai 309 control plant. Twelve of the 20 lines that had some of the clones tolerant to imazethapyr were expressing AAD-12 protein, were AAD-12 PCR PTU positive, and AHAS coding region positive. Expression levels ranged from 2.3 to 1092.4 ppm of total soluble protein.

## 21.9—Field Tolerance of DDAB4101 Rice Plants to 2,4-D and Triclopyr Herbicides.

A field level tolerance trial was conducted with AAD-12 (v1) event pDAB4101[20] and one wild-type rice (Clearfield 131) at Wayside, Miss. (a non-transgenic imidazolinone-resistant variety). The experimental design was a randomized complete block design with a single replication. Herbicide treatments were 2× rates of 2,4-D (dimethylamine salt) at 2240 g ae/ha and triclopyr at 560 g ae/ha plus an untreated control. Within each herbicide treatment, two rows of T<sub>1</sub> generation pDAB4101[20] and two rows of Clearfield rice were planted using a small plot drill with 8-inch row spacing. The pDAB4101[20] rice contained the AHAS gene as a selectable marker for the AAD-12(v1) gene. Imazethapyr was applied at the one leaf stage as selection agent to remove any AAD-12 (v1) null plants from the plots. Herbicide treatments were applied when the rice reached the 2 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual ratings of injury were taken at 7, 14 and 21 days after application.

AAD-12 (v1) event response to 2,4-D and triclopyr are shown in Table 28. The non-transformed rice line (Clearfield) was severely injured (30% at 7DAT and 35% at 15DAT) by 2,4-D at 2240 g ae/ha which is considered the 4× commercial use rate. The AAD-12 (v1) event demonstrated excellent tolerance to 2,4-D with no injury observed at 7 or 15DAT. The non-transformed rice was significantly injured (15% at 7DAT and 25% at 15DAT) by the 2× rate of triclopyr (560 g ae/ha). The AAD-12 (v1) event demonstrated excellent tolerance to the 2× rates of triclopyr with no injury observed at either 7 or 15DAT.

These results indicate that the AAD-12 (v1) transformed rice displayed a high level of resistance to 2,4-D and triclopyr at rates that caused severe visual injury to the Clearfield rice. It also demonstrates the ability to stack multiple herbicide tolerance genes with AAD-12 I multiple species to provide resistance to a wider spectrum of effective chemistries

100

## Example 22—AAD-12 (v1) in Canola

## 22.1—Canola Transformation.

The AAD-12 (v1) gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera\* 710 with *Agrobacterium*-mediated transformation and plasmid pDAB3759. The construct contained AAD-12 (v1) gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter and the EPSPS glyphosate resistance trait driven by AtUbi 10 promoter (see section 2.4).

Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C., and a photoperiod of 16 hrs light/8 hrs dark.

Hypocotyl segments (3-5 mm) were excised from 5-7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/L kinetin and 1 mg/L 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB3759. The *Agrobacterium* was grown overnight at 28° C. in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed on K1D1TC (callus induction medium containing 250 mg/L Carbenicillin and 300 mg/L Timentin) for one week or two weeks of recovery. Alternately, the segments were placed directly on selection medium K1D1H1 (above medium with 1 mg/L Herbicide). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbicide allowed the growth of the transformed cells.

Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino)ethane sulfonic acid], 5 mg/L silver nitrate, 1 mg/L Herbicide, Carbenicillin and Timentin) shoot regeneration medium. After 2-3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 3 mg/L Herbicide, Carbenicillin and Timentin) for another 2-3 weeks.

Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH5 or MES10 (MS, 0.5 gm/L MES, 5 or 10 mg/L Herbicide, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured for root induction on MSI.1 (MS with 0.1 mg/L Indolebu-

TABLE 28

AAD-12 T <sub>1</sub> generation rice plants response to 2,4-D and triclopyr under field conditions.					
Herbicide Treatment		% Visual Injury			
		7DAT		15DAT	
Active Ingredient	Rate	AAD-12 event pDAB4101[20]	Wild-type Clearfield	AAD-12 event pDAB4101[20]	Wild-type Clearfield
2,4-D	2240 GM AE/HA	0	15	0	35
Triclopyr	840 GM AE/HA	0	30	0	25
Untreated		0	0	0	0

US 10,167,483 B2

## 101

tyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

#### 22.2—Molecular Analysis: Canola Materials and Methods

##### 22.2.1—Tissue Harvesting DNA Isolation and Quantification.

Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ul.

##### 22.2.2—Polymerase Chain Reaction.

A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR AAD-12 (v1) were (SEQ ID NO:10) (forward) and (SEQ ID NO: 11) (reverse). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 2 minutes followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with AAD-12 (v1) events tested positive. Three negative control samples tested negative.

##### 22.2.3—ELISA.

Using established ELISA described in previous section, AAD-12 protein was detected in 5 different canola transformation plant events. Expression levels ranged from 14 to over 700 ppm of total soluble protein (TSP). Three different untransformed plant samples were tested in parallel with no signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. These samples were also confirmed positive by Western analysis. A summary of the results is presented in Table 29.

TABLE 29

Expression of AAD-12 (v1) in Canola plants				
Sample #	[TSP] (μg/ml)	[AAD-12] (ng/ml)	Expression (ppm TSP) (ELISA)	Western
31	5614.96	1692.12	301.36	++++
33	4988.26	2121.52	425.30	++++
38	5372.25	3879.09	722.06	++++
39	2812.77	41.36	14.71	+
40	3691.48	468.74	126.98	+++
Control 1	2736.24	0.00	0.00	—
Control 2	2176.06	0.00	0.00	—
Control 3	3403.26	0.00	0.00	—

## 102

#### 22.4—Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T<sub>0</sub> Canola.

Forty-five T<sub>0</sub> events from the transformed with the construct pDAB3759, were sent to the greenhouse over a period of time and were allowed to acclimate in the greenhouse. The plants were grown until 2-4 new, normal looking leaves had emerged (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were then treated with a lethal dose of the commercial formulations of 2,4-D Amine 4 at a rate of 560 g ae/ha. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. A lethal dose is defined as the rate that causes >95% injury to the untransformed controls.

Twenty-four of the events were tolerant to the 2,4-D DMA herbicide application. Some events did incur minor injury but recovered by 14 DAT. Events were progressed to the T<sub>1</sub> (and T<sub>2</sub> generation) by selfpollination under controlled, bagged, conditions.

#### 22.5—AAD-12 (v1) Heritability in Canola.

A 100 plant progeny test was also conducted on 11 T<sub>1</sub> lines of AAD-12 (v1). The seeds were sown and transplanted to 3-inch pots filled with Metro Mix media. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Seven out of the 11 lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi-square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species and can be stacked with one or more additional herbicide resistance genes.

#### 22.6—Verification of High 2,4-D Tolerance in T<sub>1</sub> Canola

For T<sub>1</sub> AAD-12 (v1), 5-6 mg of seed were stratified, sown, and a fine layer of Sunshine Mix #5 media was added as a top layer of soil. Emerging plants were selected with 560 g ae/ha 2,4-D at 7 and 13 days after planting.

Surviving plants were transplanted into 3-inch pots containing Metro Mix media. Surviving plants from T1 progenies, that were selected with 560 g ae/ha 2,4-D, were also transplanted into 3-inch pots filled with Metro Mix soil. At 2-4 leaf stage plants were sprayed with either 280, 560, 1120, or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to untransformed control plants. A sampling of T<sub>1</sub> event injury data 14DAT may be seen in Table 30. Data suggests that multiple events are robustly resistant to 2240 g ae/ha 2,4-D, while other events demonstrated less robust tolerance up to 1120 g ae/ha 2,4-D. Surviving plants were transplanted to 5¼" pots containing Metro Mix media and placed in the same growth conditions as before and self-pollinated to produce only homozygous seed.

TABLE 30

T <sub>1</sub> AAD-12 (v1) and untransformed control response to varying rates postemergence 2,4-D DMA applications.						
Herbicide	Untransformed Control	pDAB3759(33)	pDAB3759(18)	pDAB3759(18)	pDAB3759(18)	pDAB3759(18)
		013.001	009.001	022.001	030.001	023.001
Average % Injury 14DAT						
280 g ae/ha 2,4-D DMA	85	0	0	0	0	0
560 g ae/ha 2,4-D DMA	85	0	0	0	0	0

US 10,167,483 B2

103

104

TABLE 30-continued

T <sub>1</sub> AAD-12 (v1) and untransformed control response to varying rates postemergence 2,4-D DMA applications.						
Herbicide	Untransformed Control	pDAB3759(33) 013.001	pDAB3759(18) 009.001	pDAB3759(18) 022.001	pDAB3759(18) 030.001	pDAB3759(18) 023.001
	Average % Injury 14DAT					
1120 g ae/ha 2,4-D DMA	90	0	0	13	5	3
2240 g ae/ha 2,4-D DMA	95	1	5	83	31	6

22.7—Field Tolerance of pDAB3759 Canola Plants to 2,4-D, Dichloroprop, Triclopyr and Fluroxypyr Herbicides.

Field level tolerance trial was conducted on two AAD-12 (v1) events 3759(20)018.001 and 3759(18)030.001 and a wild-type canola (Nex710) in Fowler, Ind. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Within each herbicide treatment, single 20 ft row/event for event 3759(18)030.0011, 3759(18)018.001 and wild-type line (Nex710) were planted with a 4 row drill on 8 inch row spacing. Herbicide treatments were applied when canola reached the 4-6 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after application.

Canola response to 2,4-D, triclopyr, and fluroxypyr are shown in Table 31. The wild-type canola (Nex710) was severely injured (72% at 14DAT) by 2,4-D at 2240 g ae/ha which is considered the 4× rate. The AAD-12 (v1) events all demonstrated excellent tolerance to 2,4-D at 14DAT with an average injury of 2, 3 and 2% observed at the 1, 2 and 4× rates, respectively. The wild-type canola was severely injured (25% at 14DAT) by the 2× rate of triclopyr (840 g ae/ha). AAD-12 (v1) events demonstrated tolerance at 2× rates of triclopyr with an average of 6% injury at 14DAT across the two events. Fluroxypyr at 280 g ae/ha caused severe injury (37%) to the non-transformed line at 14DAA. AAD-12 (v1) events demonstrated increased tolerance with an average of 8% injury at 5DAT.

These results indicate that AAD-12 (v1) transformed events displayed a high level of resistance to 2,4-D, triclopyr and fluroxypyr at rates that were lethal or caused severe epinastic malformations to non-transformed canola. AAD-12 has been shown to have relative efficacy of 2,4-D>triclopyr>fluroxypyr.

Example 23—AAD-12 (v1) Stacked with Insect Resistance (IR) or Other Input Traits in any

15 Insect resistance in crops supplied by a transgenic trait is prevelant in corn and cotton production in North America and across the globe. Commercial products having combined IR and HT traits have been developed by multiple seed companies. These include Bt IR traits (e.g. Bt toxins listed at the website lifesci.sussex.ac.uk, 2006) and any or all of the HTC traits mentioned above. The value this offering brings is the ability to control multiple pest problems through genetic means in a single offering. The convenience of this offering will be restricted if weed control and insect control are accomplished independent of each other. AAD-12 (v1) alone or stacked with one or more additional HTC traits can be stacked with one or more additional input traits (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) (isb.vt.edu/cfdocs/fieldtests1.cfm, 2005) either through conventional breeding or jointly as a novel transformation event. Benefits include the convenience and flexibility described in Examples 15-20 above, together with the ability to manage insect pests and/or other agronomic stresses in addition to the improved weed control offered by AAD-12 and associated herbicide tolerance. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic issues.

40 Combined traits of IR and HT have application in most agronomic and horticultural/ornamental crops and forestry. The combination of AAD-12 and its commensurate herbicide tolerance and insect resistance afforded by any of the number of Bt or non-Bt IR genes are can be applied to the crop species listed (but not limited to) in Example 13. One skilled in the art of weed control will recognize that use of any of various commercial herbicides described in Examples 18-20, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by AAD-12 (v1) transformation and stacking with the corresponding HT trait or IR trait either by conventional breeding or genetic

TABLE 31

AAD-12 (pDAB3759) canola plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.				
Herbicide Treatment		% Visual Injury at 14 DAT		
Active Ingredient	Rate	AAD-12 event 3759(20)018.001	AAD-12 event 3759(18)030.001	Wild Type (Nex710)
2,4-D	280 GM AE/HA	0 a	0 b	0 e
2,4-D	560 GM AE/HA	0 a	0 b	15 d
2,4-D	1120 GM AE/HA	2 a	2 ab	33 bc
2,4-D	2240 GM AE/HA	3 a	3 ab	48 a
Triclopyr	840 GM AE/HA	6 a	6 ab	25 cd
Fluroxypyr	280 GM AE/HA	7 a	8 a	37 ab

Means with a column with different letters are significantly different as defined by LSD (p = 0.05).

US 10,167,483 B2

105

engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 24—AAD-12 (v1) as an In Vitro Dicot Selectable Marker

Genetic engineering of plant cell, tissue, organ, and plant or organelle such as plastid starts with the process of inserting genes of interest into plant cells using a suitable delivery method. However, when a gene is delivered to plant cells, only an extremely small percentage of cells integrate the heterogeneous gene into their genome. In order to select those few cells that have incorporated the gene of interest, researchers link a selectable or screenable “marker gene” to the gene of interest (GOI) in the vector. Cells that contain these markers are identified from the whole population of cells/tissue to which the DNA plasmid vector was delivered. By selecting those cells that express the marker gene, researchers are able to identify those few cells that may have incorporated the GOI into their genome.

There are a variety of selectable markers available to enable this selection process to obtain transgenic cells, callus, embryos, shoots and plantlets. The preferred selectable markers by the Ag-industry are herbicide markers that allow the ease of spraying compounds in the field to select the right transgenic progenies during the process of event sorting in the field situation. AAD-12 (v1) has been shown to efficiently serve as a selectable marker for whole plants transformed with the gene in the greenhouse and growth chamber (Example 7) with 2,4-D as the selection agent. Field selection is possible as well using 2,4-D in combination with the AAD-12 (v1) gene (Example 11, 22), but use in vitro for cell-level selection is complicated by the fact 2,4-D is used almost ubiquitously as a plant growth regulator in the plant tissue culture systems. Degradation of this important hormone by AAD-12 (v1) can impact the ability to use this gene as an in vitro selectable marker. Success of developing 2,4-D as a marker gene depends on identifying the right alternate plant growth regulator that can mimic the effect of 2,4-D in the respective culture system and at the same time possess the ability to be stable and not be degraded by the AAD-12 enzyme when expressed in the transgenic cells. R-dichlorprop is a close analog to 2,4-D that is not a substrate for AAD-12 (v1) and is used as a non-metabolizable auxin substitute in tobacco cell cultures allowing 2,4-D to be used at high rates as a selection agent. This fact was used in exemplifying AAD-12 (v1) could be used as a selectable marker in vitro.

24.1—(Cell Culture-Alternative Auxins.

AAD-12 (v1) degrades 2,4-D, but not R-2,4-dichlorophenoxypropionic acid (R-dichlorprop), which has at the same time the structural requirement of an auxinic growth regulator. Other non-metabolizable plant auxin mimics that may be used in cell culture include NAA (naphthalene acetic acid), IAA (indole acetic acid), dicamba, picloram, and R-mecoprop. It was investigated if it was possible to substitute R-dichlorprop and successfully maintain two different tobacco cell cultures PHL (Petite Havanna) and BY2 suspensions. Conversely, for cotton explants R-dichlorprop,

106

dicamba, and picloram were tested as alternative auxins and the embryogenic callus induction response in comparison to the standard growth regulator, 2,4-D was evaluated. Petite Havanna tobacco (PHL) and Coker cotton cotyledons were used in their experiments.

24.1.1—Tobacco Cell Suspension-2,4-D as Selection Agent.

A dose response study was conducted with both the R-dichlorprop habituated PHL cells and the R-dichlorprop habituated BY2 cells where R-dichlorprop was substituted directly for 2,4-D in culture media. Though the focus was on PHL, a dose response was also done with BY2 in case of possible future studies, as well as to help predict the dose response for PHL. For the dichlorprop habituated PHL dose response, the levels of 2,4-D used (on LSHY2C medium with R-dichlorprop) were 0 (the control), 1, 2, 3, 5, 8, 10, 12, 15, 18, 20, 40, 60, 80, 100, 110, 120 mg/L 2,4-D. There were four replications per concentration. For the R-dichlorprop habituated BY2 dose response, the levels of 2,4-D used (on LSHY2C medium) were 0 (the control), 1, 2, 3, 5, 8, 10, 20, 30, 40, mg/L 2,4-D.

The dose response was carried out showed that all the concentration of 2,4-D tested were lethal above 10 mg/L concentrations. However, there was growth in all the concentrations up to 10-mg/L 2,4-D where a slight growth of PHL suspension was observed. The growth of the suspension colonies from 1-8 mg/L 2,4-D concentrations was comparable to the growth in control treatments. The observation made in BY2 suspension cells were similar except the concentration at 10 mg/L was found to be lethal and the sub lethal concentration was 8 mg/L concentration.

24.1.2—Tobacco Cell Transformation with AAD-12 (v1) and 2,4-D Selection.

For tobacco transformation experiment, there were 11 treatments altogether: a control set plated on LS-BY2C+ dichlorprop medium, and 10 sets of LSHY2C+dichlorprop+ 2,4-D at varying concentration levels (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L). There were four replications per treatment. The plasmid DNA vector used was pDAB724, and the vector used for transformation was EHA101S strain of *Agrobacterium tumefaciens*. Four ml of PHL suspension at 0.6 OD<sup>660</sup> were mixed with 100 ul of *Agrobacterium* (either EHA101 or LBA4404 strains) suspension at 1.0 OD<sup>660</sup> in a sterile Petri plate and were mixed thoroughly and co-cultivated together in a non-shake condition at a dark growth chamber for 3 days at 25° C. After the co-cultivation period 1.5 ml of the Agro-tobacco suspension mixture was plated to the 11 set of plates above. The experiment was repeated with 13 treatments: a control of LS-BY2C+dichlorprop media (no 2,4-D), and LS-BY2C+dichlorprop+2,4-D (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L); LSHY2C+1 mg/L 2,4-D+B10 (Bialophos); LSHY2C+10 2,4-D+B10+R-dichlorprop. Again, there were four replicates per treatment, as well as a positive and negative control. All media contain 500 mg/L Carbenicillin (C) to control to contain *Agrobacterium* growth in the selection media.

The plasmid used in these experiments is pDAB724 and it has PAT selectable marker as well. So, control transformation experiments were initiated using R-dichlorprop habituated PHL in the presence of 10 mg/L bialophos following the standard protocol described above. The treatments were done side by side with 4 replicates to see if the bialophos selection in these suspension is normal.

There was little growth observed in all selection concentrations of 2,4D tested above 10 mg/L; however several fast growing colonies were found in 2, 5, an 8 mg/L 2,4-D concentration and representative sample was transferred to



US 10,167,483 B2

107

fresh selection at 10 mg/L selection to bulk the callus. Also, several putative colonies were selected in from 12, 15, 18 and 20 mg/L 2,4-D, but when compared to 10 mg/L there were only few colonies in these selection plate. Control treatment conducted with bialophos selection showed normal colony development. It appears that 10 mg/L 2,4-D is the sub-lethal and above this concentration 2,4-D appears to be lethal to the non-transformed cells. All the identified colonies were transferred to fresh medium with 10 mg/L selection and were probed for the presence of transgene by PCR as described in Example 10. The colonies selected and bulked had the transgenes as determined by PCR and expression of the genes as established by the Western analyses (as described in example 10). Several colonies were identified as actively growing and transferred to fresh selection medium with 10 mg/L 2,4-D to bulk the callus.

The bulked calluses were then transferred to higher level of 2,4-D to test the tolerance level in vitro. The levels of 2,4-D used were 20, 40, 60, 80, 100, and 120 mg/L 2,4-D. However the callus did not grow beyond 20 mg/L 2, 4-D concentrations indicating a threshold concentration higher than 20 mg/L may exist.

#### 24.2.1—Cotton Explants-Auxin Alternatives

A dose response study was initiated to test multiple auxin alternatives as a substitute for the use of 2,4-D as a growth regulator in cotton. The alternative auxin tested were 2,4-dichlorprop, dicamba, and picloram. These compounds were tested at 0.2, 2.0, and 20.0 uM concentrations respectively. 2,4-D was used as the control treatment at 0.02 uM concentration. The medium used is the base medium for cotton callus induction (Example 12). Beyond the initial phase of culture, auxin is removed from the medium to prod the tissue toward the regeneration process.

R-dichlorprop was not effective in callus induction of cotyledonary segments and appears toxic to cotton cells at the lowest concentration tested (0.02 uM). Dicamba effectively induces callus growth at all concentrations tested (0.02-20 uM) and has no apparent toxic effects in this concentration range. Callus induction with picloram increased up to a maximum when explants were treated with 0.2 uM to 20 uM. Quality of the callus was consistent with the standard 2,4-D treatment at the 2 uM picloram concentration. At the highest concentration (20 uM) 2,4-D was also inhibitory to cotton callus generation and growth.

Cotton has shown initial ability to respond effectively to alternative auxins (to 2,4-D) in culture. At high enough concentrations, 2,4-D is toxic to cotton cotyledonary explants. R-dichlorprop is surprisingly significantly more toxic to cotton than 2,4-D or other auxins. 2,4-D may be used as a selection agent and in combination with AAD-12 (v1) as the selectable marker gene. Other non-metabolizable auxin surrogates (e.g., dicamba, picloram, R-mecoprop, NAA, or IAA) would allow the use of AAD-12 as a selectable marker in dicots with 2,4-D as the selection agent.

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108

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111

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112

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## US 10,167,483 B2

## 113

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## SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 21

<210> SEQ ID NO 1

<211> LENGTH: 879

<212> TYPE: DNA

<213> ORGANISM: *Delftia acidovorans*

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catgcgctgc tgatcttccc cggccagcac ctcagcaacg accagcagat cacttttgcc      180
aaacgcttcg gcgcgatcga gcgcacgcgc ggcgggcgaca tcgtggccat ctccaatgtc      240
aaggccgatg gcacgggtgcg ccagcacagc cccgcgcagt gggacgacat gatgaaggtc      300
atcgctcgga acatggcctg gcatgccgac agcacctaca tgccggtgat ggcgcagggc      360
gcgggtgttct cggccgaagt ggtgcccgca gtgggcgggc gcacctgctt cgccgacatg      420
cgcgccgcct acgacgcgct ggacgaggcc acccgcgccc tgggtgcacca gcgctcgggc      480
cggcattcgc tgggtgtattc gcagagcaag ctgggcccac tgcagcaggc cggctcggcc      540
tacatcggtc acggcatgga caccaccgcc acgccccctg gcccgctggt caaggtgcat      600
cccgagaccg gcgcggccct cgtgctgata ggccgcccac cccatgccat cccgggcatg      660
gacgcgcgcg aatccgagcg cttcctggaa ggcctggtcg actgggcctg ccaggcgccg      720
cgggtgcatg cccaccaatg ggccgcggcg gacgtggtgg tgtgggacaa ccgctgcctg      780
ctgcaccgcg ccgagccctg ggatttcaag ctgccggggg tgatgtggca cagccgcctg      840
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<212> TYPE: PRT

<213> ORGANISM: *Delftia acidovorans*

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Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala Gly Phe Ala
          20          25          30

Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro Gly
          35          40          45

Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe Gly
          50          55          60

Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val Ala Ile Ser Asn Val
65          70          75          80
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US 10,167,483 B2

115

116

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[illegible]

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<212> TYPE: DNA
<213> ORGANISM: Delftia acidovorans
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caacatgcac	tcttgatctt	ccctgggcaa	cacctcagca	atgaccaaca	gattaccttt	180
gctaaacgct	ttggagcaat	tgagaggatt	ggcggaggtg	acattgttgc	catatccaat	240
gtcaaggcag	atggcacagt	gcgccagcac	tctcctgctg	agtgggatga	catgatgaag	300
gtcattgtgg	gcaacatggc	ctggcacgcc	gactcaacct	acatgccagt	catggctcaa	360
ggagctgtgt	tcagcgcaga	agttgtccca	gcagttgggg	gcagaacctg	ctttgtctgac	420
atgagggcag	cctacgatgc	ccttgatgag	gcaaccctg	ctcttgttca	ccaaggctct	480
gctcgtcact	cccttgtgta	ttctcagagc	aagttgggac	atgtccaaca	ggccgggtca	540
gcctacatag	gttatggcat	ggacaccact	gcaactcctc	tcagaccatt	ggteaagggtg	600
catectgaga	ctggaaggcc	cagcctcttg	atcgccgcc	atgcccatgc	catccctggc	660
atggatgcag	ctgaatcaga	gcgtctcctt	gaaggacttg	ttgactgggc	ctgccaggct	720
cccagagtcc	atgctcacca	atgggctgct	ggagatgtgg	ttgtgtggga	caaccgctgt	780
ttgtctccacc	gtgctgagcc	ctgggatttc	aagttgccac	gtgtgatgtg	gcactccaga	840
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US 10,167,483 B2

117

118

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<210> SEQ ID NO 4  
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<213> ORGANISM: Delftia acidovorans

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20 25 30  
Ala Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro  
35 40 45  
Gly Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe  
50 55 60  
Gly Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val Ala Ile Ser Asn  
65 70 75 80  
Val Lys Ala Asp Gly Thr Val Arg Gln His Ser Pro Ala Glu Trp Asp  
85 90 95  
Asp Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser  
100 105 110  
Thr Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val  
115 120 125  
Val Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala  
130 135 140  
Tyr Asp Ala Leu Asp Glu Ala Thr Arg Ala Leu Val His Gln Arg Ser  
145 150 155 160  
Ala Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln  
165 170 175  
Gln Ala Gly Ser Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr  
180 185 190  
Pro Leu Arg Pro Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser  
195 200 205  
Leu Leu Ile Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala  
210 215 220  
Glu Ser Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala  
225 230 235 240  
Pro Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp  
245 250 255  
Asp Asn Arg Cys Leu Leu His Arg Ala Glu Pro Trp Asp Phe Lys Leu  
260 265 270  
Pro Arg Val Met Trp His Ser Arg Leu Ala Gly Arg Pro Glu Thr Glu  
275 280 285  
Gly Ala Ala Leu Val  
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<210> SEQ ID NO 5  
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<213> ORGANISM: Delftia acidovorans

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caacatgctc tcctgatttt cccaggtcag cacctgtcca acgaccagca aatcactttt 180

## US 10,167,483 B2

119

120

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gcaaaacgct tcggtgcgat cgaacgtatc ggtggcgggtg atattgtggc gatctccaac	240
gtaaaagcgg atggtactgt acgtcagcac agcccggcgg agtgggacga tatgatgaag	300
gtgatcgtag gcaacatggc atggcatgct gacagcacct acatgccggt tatggcgtag	360
ggtgcggttt tctctgtga agtgggtccg gcagtgggag gtcgcacctg cttcgtagac	420
atgcgtgcag cttacgacgc gttagacgaa gctacccgcg cactgggtaca ccagcgtctt	480
gcgcgtcact ctctggtgta ttcccagagc aaactgggcc acgttcagca agcggggtcc	540
gcataatcgc gctacggtat ggataccact gcgacccgcg tgcgtccgct ggtaaaagt	600
catccggaac ccggccgtcc gtctctctg atcgccgcgc acgctcatgc gattccgggt	660
atggacgcgg cagaatccga gcgtttctct gaaggtctgg ttgattgggc ttgtcaggcg	720
ccgcgtgtgc atgtcaccga gtgggcagct ggcgacgtgg ttgtatggga taaccgctgc	780
ctgcttcacc gtgcagaacc gtgggacttt aagctgccac gtgttatgtg gcacagccgt	840
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<210> SEQ ID NO 6  
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<220> FEATURE:  
<223> OTHER INFORMATION: Chemically synthesized M13 forward sequencing primer

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gtaaaacgac ggccag	16
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<400> SEQUENCE: 8

gaacagttag acatggtcta aagg	24
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<210> SEQ ID NO 9  
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<400> SEQUENCE: 9

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<210> SEQ ID NO 10

US 10,167,483 B2

121

122

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<211> LENGTH: 22  
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<213> ORGANISM: Artificial sequence  
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coding PCR primer

<400> SEQUENCE: 10

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<213> ORGANISM: Artificial sequence  
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coding PCR primer

<400> SEQUENCE: 11

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forward coding  
region primer

<400> SEQUENCE: 12

atggctcatg ctgccctcag cc 22

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reverse coding  
region primer

<400> SEQUENCE: 13

cgggcaggcc taactccacc aa 22

<210> SEQ ID NO 14  
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<400> SEQUENCE: 14

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<400> SEQUENCE: 15

gagctcctat cactccgccg cctgctgctg cac 33

<210> SEQ ID NO 16  
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US 10,167,483 B2

123

124

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<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
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<400> SEQUENCE: 16  
  
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<223> OTHER INFORMATION: Primer br jap 3' (xhoI)  
  
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<212> TYPE: PRT  
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20 25 30  
  
Ile Glu Arg Leu Met Asp Glu Lys Ser Val Leu Val Phe Arg Gly Gln  
35 40 45  
  
Pro Leu Ser Gln Asp Gln Gln Ile Ala Phe Ala Arg Asn Phe Gly Pro  
50 55 60  
  
Leu Glu Gly Gly Phe Ile Lys Val Asn Gln Arg Pro Ser Arg Phe Lys  
65 70 75 80  
  
Tyr Ala Glu Leu Ala Asp Ile Ser Asn Val Ser Leu Asp Gly Lys Val  
85 90 95  
  
Ala Gln Arg Asp Ala Arg Glu Val Val Gly Asn Phe Ala Asn Gln Leu  
100 105 110  
  
Trp His Ser Asp Ser Ser Phe Gln Gln Pro Ala Ala Arg Tyr Ser Met  
115 120 125  
  
Leu Ser Ala Val Val Val Pro Pro Ser Gly Gly Asp Thr Glu Phe Cys  
130 135 140  
  
Asp Met Arg Ala Ala Tyr Asp Ala Leu Pro Arg Asp Leu Gln Ser Glu  
145 150 155 160  
  
Leu Glu Gly Leu Arg Ala Glu His Tyr Ala Leu Asn Ser Arg Phe Leu  
165 170 175  
  
Leu Gly Asp Thr Asp Tyr Ser Glu Ala Gln Arg Asn Ala Met Pro Pro  
180 185 190  
  
Val Asn Trp Pro Leu Val Arg Thr His Ala Gly Ser Gly Arg Lys Phe  
195 200 205  
  
Leu Phe Ile Gly Ala His Ala Ser His Val Glu Gly Leu Pro Val Ala  
210 215 220  
  
Glu Gly Arg Met Leu Leu Ala Glu Leu Leu Glu His Ala Thr Gln Arg  
225 230 235 240  
  
Glu Phe Val Tyr Arg His Arg Trp Asn Val Gly Asp Leu Val Met Trp  
245 250 255  
  
Asp Asn Arg Cys Val Leu His Arg Gly Arg Arg Tyr Asp Ile Ser Ala  
260 265 270

US 10,167,483 B2

125

126

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Arg Arg Glu Leu Arg Arg Ala Thr Thr Leu Asp Asp Ala Val Val  
275 280 285

<210> SEQ ID NO 19  
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<212> TYPE: PRT  
<213> ORGANISM: Bradyrhizobium japonicum USDA 110

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Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu  
20 25 30  
Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln  
35 40 45  
Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln  
50 55 60  
Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg  
65 70 75 80  
Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys  
85 90 95  
Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys  
100 105 110  
Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser  
115 120 125  
Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe  
130 135 140  
Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala  
145 150 155 160  
Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly  
165 170 175  
Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys  
180 185 190  
Pro Val Leu Gln Arg Leu Val Arg Thr His Pro Val His Arg Arg Lys  
195 200 205  
Ser Leu Tyr Leu Ser Ser His Ala Gly Lys Ile Ala Ser Met Ser Val  
210 215 220  
Pro Glu Gly Arg Leu Leu Leu Arg Asp Leu Asn Glu His Ala Thr Gln  
225 230 235 240  
Pro Glu Phe Val Tyr Val His Lys Trp Lys Leu His Asp Leu Val Met  
245 250 255  
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Gln Pro Arg Asp Met Arg Arg Ala Thr Val Ala Gly Thr Glu Pro Thr  
275 280 285

Val

<210> SEQ ID NO 20  
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<400> SEQUENCE: 20

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1 5 10 15

US 10,167,483 B2

127

128

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Val	Gln	Pro	Leu	Thr	Gly	Val	Leu	Gly	Ala	Glu	Ile	Thr	Gly	Val	Asp
			20					25					30		
Leu	Arg	Glu	Pro	Leu	Asp	Asp	Ser	Thr	Trp	Asn	Glu	Ile	Leu	Asp	Ala
		35					40					45			
Phe	His	Thr	Tyr	Gln	Val	Ile	Tyr	Phe	Pro	Gly	Gln	Ala	Ile	Thr	Asn
		50				55					60				
Glu	Gln	His	Ile	Ala	Phe	Ser	Arg	Arg	Phe	Gly	Pro	Val	Asp	Pro	Val
65					70					75					80
Pro	Leu	Leu	Lys	Ser	Ile	Glu	Gly	Tyr	Pro	Glu	Val	Gln	Met	Ile	Arg
				85					90					95	
Arg	Glu	Ala	Asn	Glu	Ser	Gly	Arg	Val	Ile	Gly	Asp	Asp	Trp	His	Thr
			100					105					110		
Asp	Ser	Thr	Phe	Leu	Asp	Ala	Pro	Pro	Ala	Ala	Val	Val	Met	Arg	Ala
			115					120					125		
Ile	Asp	Val	Pro	Glu	His	Gly	Gly	Asp	Thr	Gly	Phe	Leu	Ser	Met	Tyr
	130					135					140				
Thr	Ala	Trp	Glu	Thr	Leu	Ser	Pro	Thr	Met	Gln	Ala	Thr	Ile	Glu	Gly
145					150					155					160
Leu	Asn	Val	Val	His	Ser	Ala	Thr	Arg	Val	Phe	Gly	Ser	Leu	Tyr	Gln
				165					170					175	
Ala	Gln	Asn	Arg	Arg	Phe	Ser	Asn	Thr	Ser	Val	Lys	Val	Met	Asp	Val
			180					185					190		
Asp	Ala	Gly	Asp	Arg	Glu	Thr	Val	His	Pro	Leu	Val	Val	Thr	His	Pro
		195					200					205			
Gly	Ser	Gly	Arg	Lys	Gly	Leu	Tyr	Val	Asn	Gln	Val	Tyr	Cys	Gln	Arg
	210					215					220				
Ile	Glu	Gly	Met	Thr	Asp	Ala	Glu	Ser	Lys	Pro	Leu	Leu	Gln	Phe	Leu
225					230					235					240
Tyr	Glu	His	Ala	Thr	Arg	Phe	Asp	Phe	Thr	Cys	Arg	Val	Arg	Trp	Lys
			245					250						255	
Lys	Asp	Gln	Val	Leu	Val	Trp	Asp	Asn	Leu	Cys	Thr	Met	His	Arg	Ala
			260					265					270		
Val	Pro	Asp	Tyr	Ala	Gly	Lys	Phe	Arg	Tyr	Leu	Thr	Arg	Thr	Thr	Val
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Gly	Gly	Val	Arg	Pro	Ala	Arg									
	290					295									

&lt;210&gt; SEQ ID NO 21

&lt;211&gt; LENGTH: 283

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: unknown

&lt;220&gt; FEATURE:

&lt;223&gt; OTHER INFORMATION: tauD

&lt;400&gt; SEQUENCE: 21

Met	Ser	Glu	Arg	Leu	Ser	Ile	Thr	Pro	Leu	Gly	Pro	Tyr	Ile	Gly	Ala
1				5					10				15		
Gln	Ile	Ser	Gly	Ala	Asp	Leu	Thr	Arg	Pro	Leu	Ser	Asp	Asn	Gln	Phe
			20					25					30		
Glu	Gln	Leu	Tyr	His	Ala	Val	Leu	Arg	His	Gln	Val	Val	Phe	Leu	Arg
		35					40					45			
Asp	Gln	Ala	Ile	Thr	Pro	Gln	Gln	Gln	Arg	Ala	Leu	Ala	Gln	Arg	Phe
		50				55					60				
Gly	Glu	Leu	His	Ile	His	Pro	Val	Tyr	Pro	His	Ala	Glu	Gly	Val	Asp
65					70					75					80

US 10,167,483 B2

129

130

-continued

Glu	Ile	Ile	Val	Leu	Asp	Thr	His	Asn	Asp	Asn	Pro	Pro	Asp	Asn	Asp
			85					90					95		
Asn	Trp	His	Thr	Asp	Val	Thr	Phe	Ile	Glu	Thr	Pro	Pro	Ala	Gly	Ala
			100					105					110		
Ile	Leu	Ala	Ala	Lys	Glu	Leu	Pro	Ser	Thr	Gly	Gly	Asp	Thr	Leu	Trp
			115				120					125			
Thr	Ser	Gly	Ile	Ala	Ala	Tyr	Glu	Ala	Leu	Ser	Val	Pro	Phe	Arg	Gln
			130				135					140			
Leu	Leu	Ser	Gly	Leu	Arg	Ala	Glu	His	Asp	Phe	Arg	Lys	Ser	Phe	Pro
					150					155					160
Glu	Tyr	Lys	Tyr	Arg	Lys	Thr	Glu	Glu	Glu	His	Gln	Arg	Trp	Arg	Glu
				165					170					175	
Ala	Val	Ala	Lys	Asn	Pro	Pro	Leu	Leu	His	Pro	Val	Val	Arg	Thr	His
			180					185					190		
Pro	Val	Ser	Gly	Lys	Gln	Ala	Leu	Phe	Val	Asn	Glu	Gly	Phe	Thr	Thr
			195				200					205			
Arg	Ile	Val	Asp	Val	Ser	Glu	Lys	Glu	Ser	Glu	Ala	Leu	Leu	Ser	Phe
						215					220				
Leu	Phe	Ala	His	Ile	Thr	Lys	Pro	Glu	Phe	Gln	Val	Arg	Trp	Arg	Trp
					230					235					240
Gln	Pro	Asn	Asp	Ile	Ala	Ile	Trp	Asp	Asn	Arg	Val	Thr	Gln	His	Tyr
				245					250					255	
Ala	Asn	Ala	Asp	Tyr	Leu	Pro	Gln	Arg	Arg	Ile	Met	His	Arg	Ala	Thr
			260					265					270		
Ile	Leu	Gly	Asp	Lys	Pro	Phe	Tyr	Arg	Ala	Gly					
			275				280								

We claim:

1. An expression cassette for expression in a plant cell, comprising a polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein said polynucleotide encodes a protein that catalyzes degradation of phenoxy auxin and pyridyloxy auxin herbicides, wherein the polynucleotide that encodes said protein comprises SEQ ID NO:3.

2. The expression cassette of claim 1, wherein said polynucleotide comprises plant codons for expression in said plant cell.

3. The expression cassette of claim 1, wherein said plant promoter or said plant virus promoter is selected from a cassava vein mosaic virus promoter, a CaMV 35S promoter, a Figwort Mosaic Virus promoter, a rice actin promoter, a phaseolin promoter, an *Arabidopsis thaliana* Ubiquitin 10 promoter, a maize ubiquitin promoter, an *Arabidopsis thaliana* Act2 promoter, an *Arabidopsis thaliana* Ubiquitin 11 promoter, and an *Arabidopsis thaliana* Ubiquitin 3 promoter.

4. A method of controlling herbicide resistance in weeds in a field, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of crop plants comprising the expression cassette of claim 1.

5. A method of selecting at least one transgenic plant that is resistant or tolerant to both phenoxy auxin and pyridyloxy auxin herbicides, said method comprising applying a herbicidically effective amount of an aryloxyalkanoate herbicide to a plurality of plants in a field, wherein said plurality of plants in said field comprises at least one plant that is stably

35

transformed with the expression cassette of claim 1 and is able to grow in said effective amount of the aryloxyalkanoate herbicide, and identifying said plant that is stably transformed with said expression cassette.

40     **6.** The method of claim 4, wherein said plurality of crop  
plants are dicots.

7. The method of claim 6, wherein said dicots are selected from the group consisting of soybean plants, cotton plants, canola plants, sugar beet plants, tomato plants, and sunflower plants.

8. The method of claim 4, wherein said method comprises applying to said weeds at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, and wherein said pyridyloxy auxin 50 herbicide comprises one or more pyridyloxyacetate herbicides.

9. The method of claim 8, wherein said pyridyloxyacetate herbicide(s) is selected from the group consisting of triclopyr and fluroxypyr.

55 10. The method of claim 4, wherein said applying step comprises applying said herbicide to said crop plants within 14 days of planting a seed in said field, wherein said seed comprises an expression cassette comprising a polynucleotide operably linked to a heterologous plant promoter or a  
60 plant virus promoter, wherein said polynucleotide encodes a protein that catalyzes degradation of phenoxy auxin and pyridyloxy auxin herbicides, wherein the polynucleotide that encodes said protein comprises SEQ ID NO:3.

11. The method of claim 4, wherein said plurality of crop  
65 plants further comprises a second polynucleotide encoding a  
second protein that confers said crop plants with resistance  
to at least one other herbicide, wherein said second poly-

US 10,167,483 B2

131

nucleotide is heterologous, and said method further comprises applying said at least one other herbicide to least a portion of said field.

12. The method of claim 11, wherein said herbicides are applied sequentially or concurrently.

13. The method of claim 11, wherein said herbicides are applied from a tank mix.

14. The method of claim 11, wherein said at least one other herbicide is selected from the group consisting of acetochlor, acifluorfen, alloxydim, amidosulfuron, aminopyralid, atrazine, beflubutamid, bispiribac, butafenacil, cafenstrole, carfentrazone, chlorimuron, chlorotoluron, cinidon-ethyl, clethodim, clodinafop, clomazone, cloproxydim, clopyralid, cloransulam, cyanazine, cyclosulfamuron, cycloxydim, cyhalofop, daimuron, dicamba, diclofop, diclosulam, diflufenican, dimethenamid, diquat, dithiopyr, diuron, ethalfluralin, fenoxaprop, flazasulfuron, florasulam, fluazifop, flucarbazone, flufenacet, flufenican, flufenpyr, flumetsulam, flumiclorac, flumioxazin, fluroxypyr, fluthiacet, fomesafen, foramsulfuron, glufosinate, glyphosate, halosafen, halosulfuron, haloxyfop, imazamethabenz, imazamox, imazapic, imazapyr, imazaquin, imazethapyr, imazosulfuron, iodosulfuron, ioxynil, isoxaben, isoxaflutole, lactofen, linuron, mefenacet, mefluidide, mesosulfuron, mesotrione, metamifop, metazachlor, metosulam, metribuzin, MSMA, napropamide, nicosulfuron, norflurazon, oryzalin, oxadiazon, oxyfluorfen, paraquat, pebulate, pendimethalin, penoxsulam, picloram, picolinafen, pinoxaden, primisulfuron, profoxydim, propanil, pyraflufen, pyrazosulfuron, pyribenzoxim, pyriminobac, pyriithiobac, pyroxasulfone, pyroxulam, quinclorac, quinmerac, quizalofop, rimsulfuron, sethoxydim, simazine, sulcotrione, sulfentrazone, sulfometuron, tefuryltrione, tembotrione, tepraloxym, terbacil, thiazopyr, thidiazuron, thiencarbazone, thifensulfuron, thiobencarb, topramezone, tralkoxydim, triasulfuron, tribenuron, triclopyr, trifloxysulfuron, trifluralin, triflusulfuron, and tritosulfuron.

15. The method of claim 4, wherein said weeds are glyphosate-resistant.

16. The method of claim 15, wherein said crop plants are glyphosate tolerant crop plants.

132

17. The method of claim 4, wherein said herbicide is the phenoxy auxin herbicide.

18. The method of claim 17, wherein said phenoxy auxin is selected from the group consisting of MCPA and a 2,4-D herbicide.

19. A method of controlling volunteer plants in a field, wherein said method comprises applying to said volunteer plants at least one herbicide selected from the group consisting of a pyridyloxy auxin herbicide and a phenoxy auxin herbicide, said field comprising a plurality of transgenic crop plants comprising the expression cassette of claim 1.

20. The method of claim 19 wherein said volunteer plants are glyphosate-resistant and of a different species than said transgenic crop plants.

21. A polynucleotide operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein comprises SEQ ID NO:3.

22. A polynucleotide optimized for expression in a plant wherein said polynucleotide is operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein having aryloxyalkanoate dioxygenase activity, wherein the protein enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein has SEQ ID NO:3.

23. The polynucleotide of claim 22 wherein said polynucleotide is optimized for expression in a dicotyledonous plant or a monocotyledonous plant.

24. An isolated polynucleotide that is operably linked to a heterologous plant promoter or a plant virus promoter, wherein the polynucleotide encodes a protein that enzymatically degrades phenoxy auxin and pyridyloxy auxin herbicides, and wherein the polynucleotide that encodes said protein comprises SEQ ID NO:3.

25. The polynucleotide of claim 24 wherein said promoter is a cassava vein mosaic virus promoter.

\* \* \* \* \*

# **Exhibit Q**



(12) **United States Patent**  
**Wright et al.**(10) **Patent No.:** **US 11,371,055 B2**  
(45) **Date of Patent:** **\*Jun. 28, 2022**(54) **HERBICIDE RESISTANCE GENES**5,608,147 A \* 3/1997 Kaphammer ..... C12N 9/0004  
435/410(71) Applicant: **CORTEVA AGRISCIENCE LLC**,  
Indianapolis, IN (US)5,637,489 A 6/1997 Strauch et al.  
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250/559.16(\*) Notice: Subject to any disclaimer, the term of this  
patent is extended or adjusted under 35  
U.S.C. 154(b) by 217 days.This patent is subject to a terminal dis-  
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(21) Appl. No.: **15/468,494**(22) Filed: **Mar. 24, 2017**(65) **Prior Publication Data**

US 2017/0211087 A1 Jul. 27, 2017

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continuation of application No. 13/647,081, filed on  
Oct. 8, 2012, now Pat. No. 8,916,752, which is a  
continuation of application No. 12/091,896, filed as  
application No. PCT/US2006/042133 on Oct. 27,  
2006, now Pat. No. 8,283,522.(60) Provisional application No. 60/731,044, filed on Oct.  
28, 2005.(51) **Int. Cl.****C12N 15/82** (2006.01)**C12N 9/02** (2006.01)(52) **U.S. Cl.**CPC ..... **C12N 15/8274** (2013.01); **C12N 9/0069**  
(2013.01); **C12N 9/0071** (2013.01); **C12N**  
**15/8275** (2013.01); **C12Y 113/11** (2013.01)(58) **Field of Classification Search**CPC ..... C12N 9/0071  
See application file for complete search history.(56) **References Cited**

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LLP(57) **ABSTRACT**

The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The preferred enzyme and gene for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide tolerant crop trait and selectable marker opportunities.

**33 Claims, 3 Drawing Sheets****Specification includes a Sequence Listing.**

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Page 2

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**US 11,371,055 B2**

Page 6

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U.S. Patent

Jun. 28, 2022

Sheet 1 of 3

US 11,371,055 B2

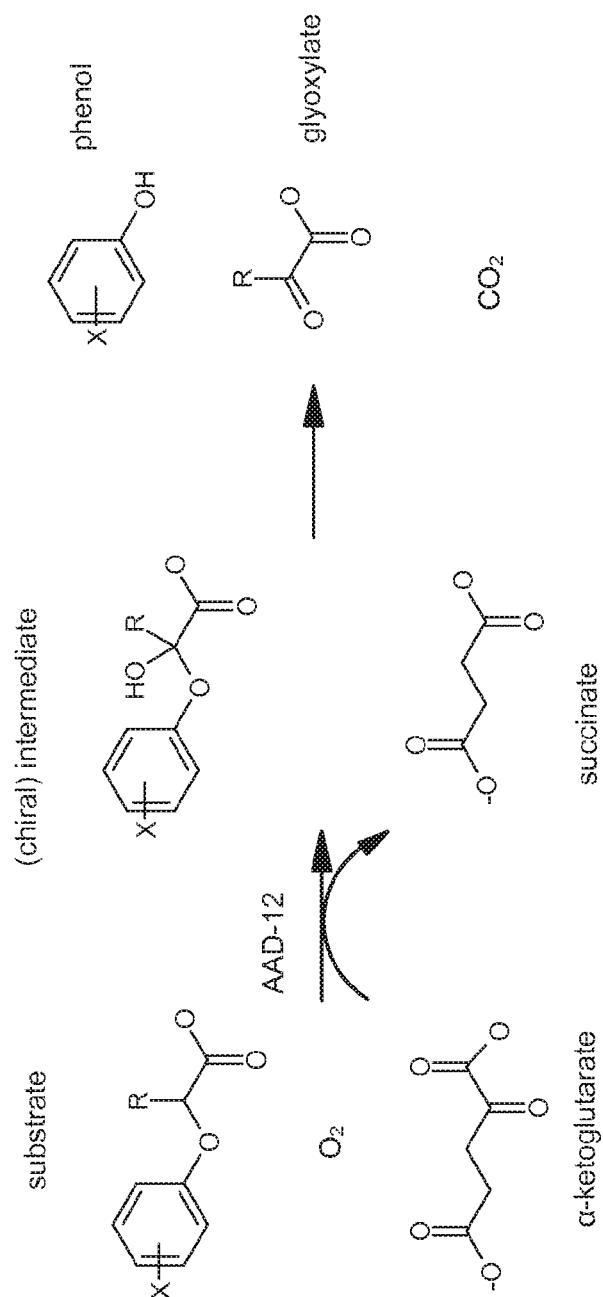


FIG. 1

## U.S. Patent

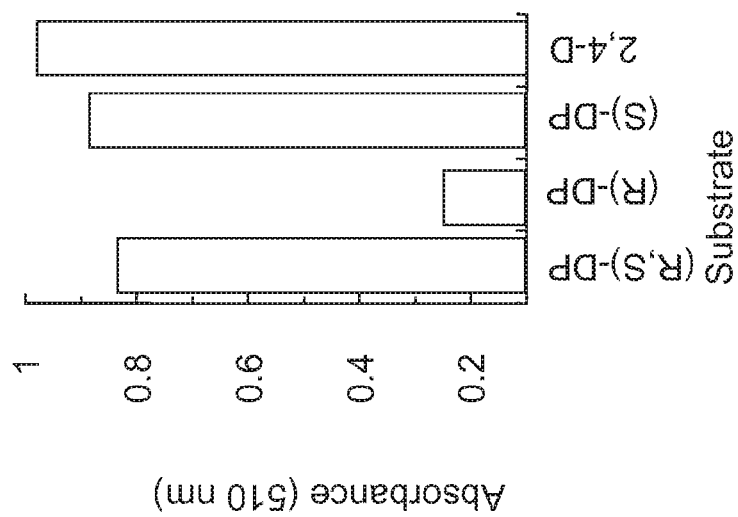
**Jun. 28, 2022**

Sheet 2 of 3

US 11,371,055 B2

	10	20	30	40	50	60	70	80
AAD-12	1	...	...	...	...	...	...	...
ttda	1	...	...	...	...	...	...	...
AAD-2	1	...	...	...	...	...	...	...
AAD-1	1	...	...	...	...	...	...	...
taud	1	...	...	...	...	...	...	...
AAD-12	64	...	...	...	...	...	...	...
ttda	67	...	...	...	...	...	...	...
AAD-2	69	...	...	...	...	...	...	...
AAD-1	75	...	...	...	...	...	...	...
taud	65	...	...	...	...	...	...	...
AAD-12	141	...	...	...	...	...	...	...
ttda	147	...	...	...	...	...	...	...
AAD-2	148	...	...	...	...	...	...	...
AAD-1	144	...	...	...	...	...	...	...
taud	132	...	...	...	...	...	...	...
AAD-12	216	...	...	...	...	...	...	...
ttda	217	...	...	...	...	...	...	...
AAD-2	218	...	...	...	...	...	...	...
AAD-1	224	...	...	...	...	...	...	...
taud	209	...	...	...	...	...	...	...

## 2. FIG.

**U.S. Patent****Jun. 28, 2022****Sheet 3 of 3****US 11,371,055 B2****FIG. 3**

US 11,371,055 B2

1

## HERBICIDE RESISTANCE GENES

## CROSS REFERENCES

This application is a continuation application of U.S. Ser. No. 14/491,197, filed Sep. 19, 2014, which is a continuation application of U.S. Ser. No. 13/647,081, filed Oct. 8, 2012, now U.S. Pat. No. 8,916,752, which is a continuation of U.S. Pat. No. 8,283,522 with Ser. No. 12/091,856, filed on Nov. 3, 2008 which claims the benefit of PCT International Application Serial No. PCT/US2006/042133, filed Oct. 27, 2006, which claims the benefit of U.S. Provisional Application Ser. No. 60/731,044, filed Oct. 28, 2005, the disclosures each of which are expressly incorporated herein by reference.

## BACKGROUND OF THE INVENTION

Weeds can quickly deplete soil of valuable nutrients needed by crops and other desirable plants. There are many different types of herbicides presently used for the control of weeds. One extremely popular herbicide is glyphosate.

Crops, such as corn, soybeans, canola, cotton, sugar beets, wheat, turf, and rice, have been developed that are resistant to glyphosate. Thus, fields with actively growing glyphosate resistant soybeans, for example, can be sprayed to control weeds without significantly damaging the soybean plants.

With the introduction of genetically engineered, glyphosate tolerant crops (GTCs) in the mid-1990's, growers were enabled with a simple, convenient, flexible, and inexpensive tool for controlling a wide spectrum of broadleaf and grass weeds unparalleled in agriculture. Consequently, producers were quick to adopt GTCs and in many instances abandon many of the accepted best agronomic practices such as crop rotation, herbicide mode of action rotation, tank mixing, incorporation of mechanical with chemical and cultural weed control. Currently glyphosate tolerant soybean, cotton, corn, and canola are commercially available in the United States and elsewhere in the Western Hemisphere. Alfalfa was the first perennial GTC introduced, furthering the opportunity for repeated use of glyphosate on the same crop and fields repeatedly over a period of years. More GTCs (e.g., wheat, rice, sugar beets, turf, etc.) are poised for introduction pending global market acceptance. Many other glyphosate resistant species are in experimental to development stages (e.g., sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, *petunia*, and begonias; see "isb.vt.edu/cfdocs/fieldtests1.cfm, 2005" website). Additionally, the cost of glyphosate has dropped dramatically in recent years to the point that few conventional weed control programs can effectively compete on price and performance with glyphosate GTC systems.

Glyphosate has been used successfully in burndown and other non-crop areas for total vegetation control for more than 15 years. In many instances, as with GTCs, glyphosate has been used 1-3 times per year for 3, 5, 10, up to 15 years in a row. These circumstances have led to an over-reliance on glyphosate and GTC technology and have placed a heavy selection pressure on native weed species for plants that are naturally more tolerant to glyphosate or which have developed a mechanism to resist glyphosate's herbicidal activity.

Extensive use of glyphosate-only weed control programs is resulting in the selection of glyphosate-resistant weeds, and is selecting for the propagation of weed species that are inherently more tolerant to glyphosate than most target

2

species (i.e., weed shifts). (Powles and Preston, 2006, Ng et al., 2003; Simarmata et al., 2003; Lorraine-Colwill et al., 2003; Sfiligoj, 2004; Miller et al., 2003; Heap, 2005; Murphy et al., 2002; Martin et al., 2002.) Although glyphosate has been widely used globally for more than 15 years, only a handful of weeds have been reported to have developed resistance to glyphosate (Heap, 2005); however, most of these have been identified in the past five years. Resistant weeds include both grass and broadleaf species—*Lolium rigidum*, *Lolium multiflorum*, *Eleusine indica*, *Sorghum halepense*, *Ambrosia artemisiifolia*, *Conyza canadensis*, *Conyza bonariensis*, *Plantago lanceolata*, *Amaranthus palmeri*, and *Amaranthus rudis*. Additionally, weeds that had previously not been an agronomic problem prior to the wide use of GTCs are now becoming more prevalent and difficult to control in the context of GTCs, which comprise >80% of U.S. cotton and soybean acres and >20% of U.S. corn acres (Gianessi, 2005). These weed shifts are occurring predominantly with (but not exclusively) difficult-to-control broadleaf weeds. Some examples include *Ipomoea*, *Amaranthus*, *Chenopodium*, *Taraxacum*, and *Commelina* species.

In areas where growers are faced with glyphosate resistant weeds or a shift to more difficult-to-control weed species, growers can compensate for glyphosate's weaknesses by tank mixing or alternating with other herbicides that will control the missed weeds. One popular and efficacious tankmix partner for controlling broadleaf escapes in many instances has been 2,4-dichlorophenoxyacetic acid (2,4-D). 2,4-D has been used agronomically and in non-crop situations for broad spectrum, broadleaf weed control for more than 60 years. Individual cases of more tolerant species have been reported, but 2,4-D remains one of the most widely used herbicides globally. A limitation to further use of 2,4-D is that its selectivity in dicot crops like soybean or cotton is very poor, and hence 2,4-D is not typically used on (and generally not near) sensitive dicot crops. Additionally, 2,4-D's use in grass crops is somewhat limited by the nature of crop injury that can occur. 2,4-D in combination with glyphosate has been used to provide a more robust burndown treatment prior to planting no-till soybeans and cotton; however, due to these dicot species' sensitivity to 2,4-D, these burndown treatments must occur at least 14-30 days prior to planting (Agrilience, 2005).

2,4-D is in the phenoxy acid class of herbicides, as is MCPA. 2,4-D has been used in many monocot crops (such as corn, wheat, and rice) for the selective control of broadleaf weeds without severely damaging the desired crop plants. 2,4-D is a synthetic auxin derivative that acts to deregulate normal cell-hormone homeostasis and impede balanced, controlled growth; however, the exact mode of action is still not known. Triclopyr and fluroxypyr are pyridyloxyacetic acid herbicides whose mode of action is as a synthetic auxin, also.

These herbicides have different levels of selectivity on certain plants (e.g., dicots are more sensitive than grasses). Differential metabolism by different plants is one explanation for varying levels of selectivity. In general, plants metabolize 2,4-D slowly, so varying plant response to 2,4-D may be more likely explained by different activity at the target site(s) (WSSA, 2002). Plant metabolism of 2,4-D typically occurs via a two-phase mechanism, typically hydroxylation followed by conjugation with amino acids or glucose (WSSA, 2002).

Over time, microbial populations have developed an alternative and efficient pathway for degradation of this particular xenobiotic, which results in the complete miner-

US 11,371,055 B2

3

alization of 2,4-D. Successive applications of the herbicide select for microbes that can utilize the herbicide as a carbon source for growth, giving them a competitive advantage in the soil. For this reason, 2,4-D currently formulated has a relatively short soil half-life, and no significant carryover effects to subsequent crops are encountered. This adds to the herbicidal utility of 2,4-D.

One organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzymatic step in the mineralization pathway is *tfdA*. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. *TfdA* catalyzes the conversion of 2,4-D acid to dichlorophenol (DCP) via an  $\alpha$ -ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). DCP has little herbicidal activity compared to 2,4-D. *TfdA* has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al. (1989), Lyon et al. (1989), Lyon (1993), and U.S. Pat. No. 5,608,147).

A large number of *tfdA*-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are similar to *tfdA* (>85% amino acid identity) and have similar enzymatic properties to *tfdA*. However, there are a number of homologues that have a significantly lower identity to *tfdA* (25-50%), yet have the characteristic residues associated with  $\alpha$ -ketoglutarate dioxygenase  $\text{Fe}^{+2}$  dioxygenases. It is therefore not obvious what the substrate specificities of these divergent dioxygenases are.

One unique example with low homology to *tfdA* (31% amino acid identity) is *sdpA* from *Delftia acidovorans* (Kohler et al., 1999, Westendorf et al., 2002, Westendorf et al., 2003). This enzyme has been shown to catalyze the first step in (S)-dichlorprop (and other (S)-phenoxypropionic acids) as well as 2,4-D (a phenoxyacetic acid) mineralization (Westendorf et al., 2003). Transformation of this gene into plants, has not heretofore been reported.

Development of new herbicide-tolerant crop (HTC) technologies has been limited in success due largely to the efficacy, low cost, and convenience of GTCs. Consequently, a very high rate of adoption for GTCs has occurred among producers. This created little incentive for developing new HTC technologies.

Aryloxyalkanoate chemical substructures are a common entity of many commercialized herbicides including the phenoxyacetate auxins (such as 2,4-D and dichlorprop), pyridyloxyacetate auxins (such as fluroxypyr and triclopyr), aryloxyphenoxypropionates (AOPP) acetyl-coenzyme A carboxylase (ACCase) inhibitors (such as haloxyfop, quizalofop, and diclofop), and 5-substituted phenoxyacetate protoporphyrinogen oxidase IX inhibitors (such as pyraflufen and flumiclorac). However, these classes of herbicides are all quite distinct, and no evidence exists in the current literature for common degradation pathways among these chemical classes. A multifunctional enzyme for the degradation of herbicides covering multiple modes of action has recently been described (PCT US/2005/014737; filed May 2, 2005). Another unique multifunctional enzyme and potential uses are described hereafter.

4

## BRIEF SUMMARY OF THE INVENTION

The subject invention provides novel plants that are not only resistant to 2,4-D, but also to pyridyloxyacetate herbicides. Heretofore, there was no expectation or suggestion that a plant with both of these advantageous properties could be produced by the introduction of a single gene. The subject invention also includes plants that produce one or more enzymes of the subject invention "stacked" together with one or more other herbicide resistance genes, including, but not limited to, glyphosate-, ALS- (imidazolinone, sulfonylurea), aryloxyalkanoate-, HPPD-, PPO-, and glufosinate-resistance genes, so as to provide herbicide-tolerant plants compatible with broader and more robust weed control and herbicide resistance management options. The present invention further includes methods and compositions utilizing homologues of the genes and proteins exemplified herein.

In some embodiments, the invention provides monocot and dicot plants tolerant to 2,4-D, MCPA, triclopyr, fluroxypyr, and one or more commercially available herbicides (e.g., glyphosate, glufosinate, paraquat, ALS-inhibitors (e.g., sulfonylureas, imidazolinones, triazopyrimidine sulfonanilides, et al), HPPD inhibitors (e.g., mesotrione, isoxaflutole, et al.), dicamba, bromoxynil, aryloxyphenoxypropionates, and others). Vectors comprising nucleic acid sequences responsible for such herbicide tolerance are also disclosed, as are methods of using such tolerant plants and combinations of herbicides for weed control and prevention of weed population shifts. The subject invention enables novel combinations of herbicides to be used in new ways. Furthermore, the subject invention provides novel methods of preventing the development of, and controlling, strains of weeds that are resistant to one or more herbicides such as glyphosate. The subject invention enables novel uses of novel combinations of herbicides and crops, including pre-plant application to an area to be planted immediately prior to planting with seed for plants that would otherwise be sensitive to that herbicide (such as 2,4-D).

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known *tfdA* proteins, for example. More specifically, the subject invention relates to the use of an enzyme that is capable of degrading both 2,4-D and pyridyloxyacetate herbicides. No  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of both the phenoxyacetate and pyridyloxyacetates auxin herbicides. The preferred enzyme and gene for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase). This highly novel discovery is the basis of significant herbicide-tolerant crop (HTC) trait and selectable marker opportunities. Plants of the subject invention can be resistant throughout their entire life cycle.

There was no prior motivation to produce plants comprising an AAD-12 gene (preferably an AAD-12 polynucleotide that has a sequence optimized for expression in one or more types of plants, as exemplified herein), and there was no expectation that such plants could effectively produce an

US 11,371,055 B2

5

AAD-12 enzyme to render the plants resistant a phenoxy-acetic acid herbicide (such as 2,4-D) and/or one or more pyridyloxyacetates herbicides such as triclopyr and fluroxypyr. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxyacetate auxin and/or pyridyloxyacetates auxin herbicides. Methods of screening proteins for these activities are within the scope of the subject invention. Thus, the subject invention includes degradation of 2,4-dichlorophenoxyacetic acid and other aryloxyalkanoate auxin herbicides by a recombinantly expressed AAD-12 enzyme. The subject invention also includes methods of controlling weeds wherein said methods comprise applying one or more pyridyloxyacetate or phenoxyacetate auxin herbicides to plants comprising an AAD-12 gene. The subject invention also provides methods of using an AAD-12 gene as a selectable marker for identifying plant cells and whole plants transformed with AAD-12, optionally including one, two, or more exogenous genes simultaneously inserted into target plant cells. Methods of the subject invention include selecting transformed cells that are resistant to appropriate levels of an herbicide. The subject invention further includes methods of preparing a polypeptide, having the biological activity of aryloxyalkanoate dioxygenase, by culturing plants and/or cells of the subject invention.

#### BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 illustrates the general chemical reaction that is catalyzed by AAD-12 enzymes of the subject invention.

FIG. 2 is an amino acid sequence alignment of an exemplified AAD-12 protein (SEQ ID NO:2), TfdA (SEQ ID NO:18), AAD-2 (SEQ ID NO:19), AAD-1 (SEQ ID NO 20), and TauD (SEQ ID NO:21).

FIG. 3 illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichloroprop.

#### BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO:1 is the nucleotide sequence of AAD-12 from *Delftia acidovorans*.

SEQ ID NO:2 is the translated protein sequence encoded by SEQ ID NO:1.

SEQ ID NO:3 is the plant optimized nucleotide sequence of AAD-12 (v).

SEQ ID NO:4 is the translated protein sequence encoded by SEQ ID NO:3.

SEQ ID NO:5 is the *E. coli* optimized nucleotide sequence of AAD-12 (v2).

SEQ ID NO:6 is the sequence of the M13 forward primer.

SEQ ID NO:7 is the sequence of the M13 reverse primer.

SEQ ID NO:8 is the sequence of the forward AAD-12 (v1) PTU primer.

SEQ ID NO:9 is the sequence of the reverse AAD-12 (v1) PTU primer.

SEQ ID NO:10 is the sequence of the forward AAD-12 (v1) coding PCR primer.

6

SEQ ID NO: 11 is the sequence of the reverse AAD-12 (v1) coding PCR primer.

SEQ ID NO:12 shows the sequence of the "sdpacodF" AAD-12 (v1) primer.

SEQ ID NO: 13 shows the sequence of the "sdpacodR" AAD-12 (v1) primer.

SEQ ID NO:14 shows the sequence of the "Nco1 of Brady" primer.

SEQ ID NO:15 shows the sequence of the "Sac1 of Brady" primer.

SEQ ID NO:16 provides the sequence of forward primer brjap 5' (speI).

SEQ ID NO:17 provides the sequence of reverse primer brjap 3' (xhoI).

SEQ ID NO:18 provides the sequence of tfdA.

SEQ ID NO:19 provides the sequence of AAD-2.

SEQ ID NO:20 provides the sequence of AAD-1.

SEQ ID NO:21 provides the sequence of tauD.

#### DETAILED DESCRIPTION OF THE INVENTION

The subject development of a 2,4-D resistance gene and subsequent resistant crops provides excellent options for controlling broadleaf, glyphosate-resistant (or highly tolerant and shifted) weed species for in-crop applications. 2,4-D is a broad-spectrum, relatively inexpensive, and robust broadleaf herbicide that would provide excellent utility for growers if greater crop tolerance could be provided in dicot and monocot crops alike. 2,4-D-tolerant transgenic dicot crops would also have greater flexibility in the timing and rate of application. An additional utility of the subject herbicide tolerance trait for 2,4-D is its utility to prevent damage to normally sensitive crops from 2,4-D drift, volatilization, inversion (or other off-site movement phenomenon), misapplication, vandalism, and the like. An additional benefit of the AAD-12 gene is that unlike all tfdA homologues characterized to date, AAD-12 is able to degrade the pyridyloxyacetates auxins (e.g., triclopyr, fluroxypyr) in addition to achiral phenoxy auxins (e.g., 2,4-D, MCPA, 4-chlorophenoxyacetic acid). See Table 1. A general illustration of the chemical reactions catalyzed by the subject AAD-12 enzyme is shown in FIG. 1. (Addition of O<sub>2</sub> is stereospecific; breakdown of intermediate to phenol and glyoxylate is spontaneous.) It should be understood that the chemical structures in FIG. 1 illustrate the molecular backbones and that various R groups and the like (such as those shown in Table 1) are included but are not necessarily specifically illustrated in FIG. 1. Multiple mixes of different phenoxy auxin combinations have been used globally to address specific weed spectra and environmental conditions in various regions. Use of the AAD-12 gene in plants affords protection to a much wider spectrum of auxin herbicides, thereby increasing the flexibility and spectra of weeds that can be controlled. The subject invention can also be used to protect from drift or other off-site synthetic auxin herbicide injury for the full breadth of commercially available phenoxy auxins. Table 1 defines commercially available pyridyloxy and phenoxy auxins and provides relevant chemical structures.



## US 11,371,055 B2

7

8

TABLE 1

Commercially available phenoxyacetate and pyridyloxyacetate auxins. Reference to phenoxy auxin and pyridyloxy auxin herbicides is generally made to the active acid but some are commercially formulated as any of a variety of corresponding ester formulations and these are likewise considered as substrates for AAD-12 enzyme in planta as general plant esterases convert these esters to the active acids in planta. Likewise reference can also be for the corresponding organic or inorganic salt of the corresponding acid. Possible use rate ranges can be as stand-alone treatments or in combination with other herbicides in both crop and non-crop uses.

Chemical name	CAS no	Possible use rate ranges (g ac/ha)	Preferred use rate ranges (g ac/ha)	Structure
2,4-D	94-75-7	25-4000	280-1120	
2,4,5-T	93-76-5	25-4000	25-4000	
4-CPA	122-88-3	25-4000	25-4000	
3,4-DA	588-22-7	25-4000	25-4000	
MCPA	94-74-6	25-4000	125-1550	
Triclopyr	55335-06-3	50-2000	70-840	
Fluroxypyr	69377-81-7	25-2000	35-560	

A single gene (AAD-12) has now been identified which, when genetically engineered for expression in plants, has the properties to allow the use of phenoxy auxin herbicides in plants where inherent tolerance never existed or was not sufficiently high to allow use of these herbicides. Addition-

ally, AAD-12 can provide protection in planta to pyridyloxyacetate herbicides where natural tolerance also was not sufficient to allow selectivity, expanding the potential utility of these herbicides. Plants containing AAD-12 alone now may be treated sequentially or tank mixed with one, two, or

US 11,371,055 B2

9

a combination of several phenoxy auxin herbicides. The rate for each phenoxy auxin herbicide may range from 25 to 4000 g ae/ha, and more typically from 100 to 2000 g ae/ha for the control of a broad spectrum of dicot weeds. Likewise, one, two, or a mixture of several pyridyloxyacetate auxin compounds may be applied to plants expressing AAD-12 with reduced risk of injury from said herbicides. The rate for each pyridyloxyacetate herbicide may range from 25 to 2000 g ae/ha, and more typically from 35-840 g ae/ha for the control of additional dicot weeds.

Glyphosate is used extensively because it controls a very wide spectrum of broadleaf and grass weed species. However, repeated use of glyphosate in GTCs and in non-crop applications has, and will continue to, select for weed shifts to naturally more tolerant species or glyphosate-resistant biotypes. Tankmix herbicide partners used at efficacious rates that offer control of the same species but having different modes of action is prescribed by most herbicide resistance management strategies as a method to delay the appearance of resistant weeds. Stacking AAD-12 with a glyphosate tolerance trait (and/or with other herbicide-tolerance traits) could provide a mechanism to allow for the control of glyphosate resistant dicot weed species in GTCs by enabling the use of glyphosate, phenoxy auxin(s) (e.g., 2,4-D) and pyridyloxyacetates auxin herbicides (e.g., triclopyr)-selectively in the same crop. Applications of these herbicides could be simultaneously in a tank mixture comprising two or more herbicides of different modes of action; individual applications of single herbicide composition in sequential applications as pre-plant, preemergence, or postemergence and split timing of applications ranging from approximately 2 hours to approximately 3 months; or, alternatively, any combination of any number of herbicides representing each chemical class can be applied at any timing within about 7 months of planting the crop up to harvest of the crop (or the preharvest interval for the individual herbicide, whichever is shortest).

It is important to have flexibility in controlling a broad spectrum of grass and broadleaf weeds in terms of timing of application, rate of individual herbicides, and the ability to control difficult or resistant weeds. Glyphosate applications in a crop with a glyphosate resistance gene/AAD-12 stack could range from about 250-2500 g ae/ha; phenoxy auxin herbicide(s) (one or more) could be applied from about 25-4000 g ae/ha; and pyridyloxyacetates auxin herbicide(s) (one or more) could be applied from 25-2000 g ae/ha. The optimal combination(s) and timing of these application(s) will depend on the particular situation, species, and environment, and will be best determined by a person skilled in the art of weed control and having the benefit of the subject disclosure.

Plantlets are typically resistant throughout the entire growing cycle. Transformed plants will typically be resistant to new herbicide application at any time the gene is expressed. Tolerance is shown herein to 2,4-D across the life cycle using the constitutive promoters tested thus far (primarily CsVMV and AtUbi 10). One would typically expect this, but it is an improvement upon other non-metabolic activities where tolerance can be significantly impacted by the reduced expression of a site of action mechanism of resistance, for example. One example is Roundup Ready cotton, where the plants were tolerant if sprayed early, but if sprayed too late the glyphosate concentrated in the meristems (because it is not metabolized and is translocated); viral promoters Monsanto used are not well expressed in the flowers. The subject invention provides an improvement in these regards.

10

Herbicide formulations (e.g., ester, acid, or salt formulation; or soluble concentrate, emulsifiable concentrate, or soluble liquid) and tankmix additives (e.g., adjuvants, surfactants, drift retardants, or compatibility agents) can significantly affect weed control from a given herbicide or combination of one or more herbicides. Any combination of these with any of the aforementioned herbicide chemistries is within the scope of this invention.

One skilled in the art would also see the benefit of combining two or more modes of action for increasing the spectrum of weeds controlled and/or for the control of naturally more tolerant or resistant weed species. This could also extend to chemistries for which herbicide tolerance was enabled in crops through human involvement (either transgenically or non-transgenically) beyond GTCs. Indeed, traits encoding glyphosate resistance (e.g., resistant plant or bacterial EPSPS, glyphosate oxidoreductase (GOX), GAT), glufosinate resistance (e.g., Pat, bar), acetolactate synthase (ALS)-inhibiting herbicide resistance (e.g., imidazolinone, sulfonylurea, triazolopyrimidine sulfonanilide, pyrimidinylthiobenzoates, and other chemistries=AHAS, Csr1, SurA, et al.), bromoxynil resistance (e.g., Bxn), resistance to inhibitors of HPPD (4-hydroxyphenyl-pyruvate-dioxygenase) enzyme, resistance to inhibitors of phytoene desaturase (PDS), resistance to photosystem II inhibiting herbicides (e.g., psbA), resistance to photosystem I inhibiting herbicides, resistance to protoporphyrinogen oxidase IX (PPO)-inhibiting herbicides (e.g., PPO-1), resistance to phenylurea herbicides (e.g., CYP76B1), dicamba-degrading enzymes (see, e.g., US 20030135879), and others could be stacked alone or in multiple combinations to provide the ability to effectively control or prevent weed shifts and/or resistance to any herbicide of the aforementioned classes. In vivo modified EPSPS can be used in some preferred embodiments, as well as Class I, Class II, and Class III glyphosate resistance genes.

Regarding additional herbicides, some additional preferred ALS inhibitors include but are not limited to the sulfonylureas (such as chlorsulfuron, halosulfuron, nicosulfuron, sulfometuron, sulfosulfuron, trifloxysulfuron), imidazolinones (such as imazamox, imazethapyr, imazaquin), triazolopyrimidine sulfonanilides (such as cloransulam-methyl, diclosulam, florasulam, flumetsulam, metosulam, and penoxsulam), pyrimidinylthiobenzoates (such as bispyribac and pyriithobac), and flucarbazone. Some preferred HPPD inhibitors include but are not limited to mesotrione, isoxaflutole, and sulcotrione. Some preferred PPO inhibitors include but are not limited to flumiclorac, flumioxazin, flufenpyr, pyraflufen, fluthiacet, butafenacil, carfentrazone, sulfentrazone, and the diphenylethers (such as acifluorfen, fomesafen, lactofen, and oxyfluorfen).

Additionally, AAD-12 alone or stacked with one or more additional HTC traits can be stacked with one or more additional input (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) or output (e.g., increased yield, improved oil profile, improved fiber quality, et al.) traits. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic pests.

The subject invention relates in part to the identification of an enzyme that is not only able to degrade 2,4-D, but also surprisingly possesses novel properties, which distinguish the enzyme of the subject invention from previously known tfdA proteins, for example. Even though this enzyme has very low homology to tfdA, the genes of the subject invention can still be generally classified in the same overall

US 11,371,055 B2

11

family of  $\alpha$ -ketoglutarate-dependent dioxygenases. This family of proteins is characterized by three conserved histidine residues in a "HX(D/E)X<sub>23-26</sub>(T/S)X<sub>114-183</sub>HX<sub>10-13</sub>R" motif which comprises the active site. The histidines coordinate Fe<sup>+2</sup> ion in the active site that is essential for catalytic activity (Hogan et al., 2000). The preliminary in vitro expression experiments discussed herein were tailored to help select for novel attributes. These experiments also indicate the AAD-12 enzyme is unique from another disparate enzyme of the same class, disclosed in a previously filed patent application (PCT US/2005/014737; filed May 2, 2005). The AAD-1 enzyme of that application shares only about 25% sequence identity with the subject AAD-12 protein.

More specifically, the subject invention relates in part to the use of an enzyme that is not only capable of degrading 2,4-D, but also pyridyloxyacetate herbicides. No  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of different chemical classes and modes of action. Preferred enzymes and genes for use according to the subject invention are referred to herein as AAD-12 (AryloxyAlkanoate Dioxygenase) genes and proteins. As disclosed herein a peptide of SEQ ID NO: 2 is provided that is capable of degrading a phenoxy auxin herbicide and a pyridyloxy auxin herbicide. Mapping the  $\alpha$ -ketoglutarate-dependent dioxygenase motif disclosed in the previous paragraph to the sequence of SEQ ID NO: 2 reveals the AAD-12 motif of

HX<sub>109</sub>D(X)<sub>111-134</sub>T(X)<sub>136-261</sub>H(X)<sub>263-272</sub>R, wherein X<sub>109</sub> represents a single amino acid at position 109, relative to the sequence of SEQ ID NO: 2; (X)<sub>111-134</sub> represents a sequence of 24 amino acids; (X)<sub>136-261</sub> represents a sequence of 126 amino acids; and (X)<sub>263-272</sub> represents a sequence of 10 amino acids.

This invention also relates in part to the identification and use of genes encoding aryloxyalkanoate dioxygenase enzymes that are capable of degrading phenoxy auxin and pyridyloxyacetate herbicides. Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic acids, and pyridyloxyacetic acid herbicides by a recombinantly expressed AAD-12 enzyme.

The subject proteins tested positive for 2,4-D conversion to 2,4-dichlorophenol ("DCP"; herbicidally inactive) in analytical assays. Partially purified proteins of the subject invention can rapidly convert 2,4-D to DCP in vitro. An additional advantage that AAD-12 transformed plants provide is that parent herbicide(s) are metabolized to inactive forms, thereby reducing the potential for harvesting herbicidal residues in grain or stover.

The subject invention also includes methods of controlling weeds wherein said methods comprise applying a pyridyloxyacetate and/or a phenoxy auxin herbicide to plants comprising an AAD-12 gene.

In light of these discoveries, novel plants that comprise a polynucleotide encoding this type of enzyme are now provided. Heretofore, there was no motivation to produce such plants, and there was no expectation that such plants could effectively produce this enzyme to render the plants resistant to not only phenoxy acid herbicides (such as 2,4-D) but also pyridyloxyacetate herbicides. Thus, the subject invention provides many advantages that were not heretofore thought to be possible in the art.

Publicly available strains (deposited in culture collections like ATCC or DSMZ) can be acquired and screened, using techniques disclosed herein, for novel genes. Sequences disclosed herein can be used to amplify and clone the

12

homologous genes into a recombinant expression system for further screening and testing according to the subject invention.

As discussed above in the Background section, one organism that has been extensively researched for its ability to degrade 2,4-D is *Ralstonia eutropha* (Streber et al., 1987). The gene that codes for the first enzyme in the degradation pathway is tfdA. See U.S. Pat. No. 6,153,401 and GENBANK Acc. No. M16730. Tfd4 catalyzes the conversion of 2,4-D acid to herbicidally inactive DCP via an  $\alpha$ -ketoglutarate-dependent dioxygenase reaction (Smejkal et al., 2001). TfdA has been used in transgenic plants to impart 2,4-D resistance in dicot plants (e.g., cotton and tobacco) normally sensitive to 2,4-D (Streber et al., 1989; Lyon et al., 1989; Lyon et al., 1993). A large number of tfdA-type genes that encode proteins capable of degrading 2,4-D have been identified from the environment and deposited into the Genbank database. Many homologues are quite similar to tfdA (>85% amino acid identity) and have similar enzymatic properties to tfdA. However, a small collection of  $\alpha$ -ketoglutarate-dependent dioxygenase homologues are presently identified that have a low level of homology to tfd4.

The subject invention relates in part to surprising discoveries of new uses for and functions of a distantly related enzyme, sdpA, from *Delftia acidivorans* (Westendorf et al., 2002, 2003) with low homology to tfdA (31% amino acid identity). This  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme purified in its native form had previously been shown to degrade 2,4-D and S-dichloroprop (Westendorf et al., 2002 and 2003). However, no  $\alpha$ -ketoglutarate-dependent dioxygenase enzyme has previously been reported to have the ability to degrade herbicides of pyridyloxyacetate chemical class. SdpA has never been expressed in plants, nor was there any motivation to do so in part because development of new HTC technologies has been limited due largely to the efficacy, low cost, and convenience of GTCs (Devine, 2005).

In light of the novel activity, proteins and genes of the subject invention are referred to herein as AAD-12 proteins and genes. AAD-12 was presently confirmed to degrade a variety of phenoxyacetate auxin herbicides in vitro. However, this enzyme, as reported for the first time herein, was surprisingly found to also be capable of degrading additional substrates of the class of aryloxyalkanoate molecules. Substrates of significant agronomic importance include the pyridyloxyacetate auxin herbicides. This highly novel discovery is the basis of significant Herbicide Tolerant Crop (HTC) and selectable marker trait opportunities. This enzyme is unique in its ability to deliver herbicide degradative activity to a range of broad spectrum broadleaf herbicides (phenoxyacetate and pyridyloxyacetate auxins).

Thus, the subject invention relates in part to the degradation of 2,4-dichlorophenoxyacetic acid, other phenoxyacetic auxin herbicides, and pyridyloxyacetate herbicides by a recombinantly expressed aryloxyalkanoate dioxygenase enzyme (AAD-12). This invention also relates in part to identification and uses of genes encoding an aryloxyalkanoate dioxygenase degrading enzyme (AAD-12) capable of degrading phenoxy and/or pyridyloxy auxin herbicides.

The subject enzyme enables transgenic expression resulting in tolerance to combinations of herbicides that would control nearly all broadleaf weeds. AAD-12 can serve as an excellent herbicide tolerant crop (HTC) trait to stack with other HTC traits [e.g., glyphosate resistance, glufosinate resistance, ALS-inhibitor (e.g., imidazolinone, sulfonylurea, triazopyrimidine sulfonamide) resistance, bromoxynil resistance, HPPD-inhibitor resistance, PPO-inhibitor resistance, et al.], and insect resistance traits (Cry1F, Cry1Ab.

US 11,371,055 B2

13

Cry 34/45, other Bt. Proteins, or insecticidal proteins of a non-Bacillus origin, et al.) for example. Additionally, AAD-12 can serve as a selectable marker to aid in selection of primary transformants of plants genetically engineered with a second gene or group of genes.

In addition, the subject microbial gene has been redesigned such that the protein is encoded by codons having a bias toward both monocot and dicot plant usage (hemicot). *Arabidopsis*, corn, tobacco, cotton, soybean, canola, and rice have been transformed with AAD-12-containing constructs and have demonstrated high levels of resistance to both the phenoxy and pyridyloxy auxin herbicides. Thus, the subject invention also relates to “plant optimized” genes that encode proteins of the subject invention.

Oxyalkanoate groups are useful for introducing a stable acid functionality into herbicides. The acidic group can impart phloem mobility by “acid trapping,” a desirable attribute for herbicide action and therefore could be incorporated into new herbicides for mobility purposes. Aspects of the subject invention also provide a mechanism of creating HTC. There exist many potential commercial and experimental herbicides that can serve as substrates for AAD-12. Thus, the use of the subject genes can also result in herbicide tolerance to those other herbicides as well.

HTC traits of the subject invention can be used in novel combinations with other HTC traits (including but not limited to glyphosate tolerance). These combinations of traits give rise to novel methods of controlling weed (and like) species, due to the newly acquired resistance or inherent tolerance to herbicides (e.g., glyphosate). Thus, in addition to the HTC traits, novel methods for controlling weeds using herbicides, for which herbicide tolerance was created by said enzyme in transgenic crops, are within the scope of the invention.

This invention can be applied in the context of commercializing a 2,4-D resistance trait stacked with current glyphosate resistance traits in soybeans, for example. Thus, this invention provides a tool to combat broadleaf weed species shifts and/or selection of herbicide resistant broadleaf weeds, which culminates from extremely high reliance by growers on glyphosate for weed control with various crops.

The transgenic expression of the subject AAD-12 genes is exemplified in, for example, *Arabidopsis*, tobacco, soybean, cotton, rice, corn and canola. Soybeans are a preferred crop for transformation according to the subject invention. However, this invention can be utilized in multiple other monocot (such as pasture grasses or turf grass) and dicot crops like alfalfa, clover, tree species, et al. Likewise, 2,4-D (or other AAD-12-substrates) can be more positively utilized in grass crops where tolerance is moderate, and increased tolerance via this trait would provide growers the opportunity to use these herbicides at more efficacious rates and over a wider application timing without the risk of crop injury.

Still further, the subject invention provides a single gene that can provide resistance to herbicides that control broadleaf weed. This gene may be utilized in multiple crops to enable the use of a broad spectrum herbicide combination. The subject invention can also control weeds resistant to current chemicals, and aids in the control of shifting weed spectra resulting from current agronomic practices. The subject AAD-12 can also be used in efforts to effectively detoxify additional herbicide substrates to non-herbicidal forms. Thus, the subject invention provides for the development of additional HTC traits and/or selectable marker technology.

Separate from, or in addition to, using the subject genes to produce HTCs, the subject genes can also be used as

14

selectable markers for successfully selecting transformants in cell cultures, greenhouses, and in the field. There is high inherent value for the subject genes simply as a selectable marker for biotechnology projects. The promiscuity of AAD-12 for other aryloxyalkanoate auxinic herbicides provides many opportunities to utilize this gene for HTC and/or selectable marker purposes.

Proteins (and Source Isolates) of the Subject Invention. The present invention provides functional proteins. By “functional activity” (or “active”) it is meant herein that the proteins/enzymes for use according to the subject invention have the ability to degrade or diminish the activity of a herbicide (alone or in combination with other proteins). Plants producing proteins of the subject invention will preferably produce “an effective amount” of the protein so that when the plant is treated with a herbicide, the level of protein expression is sufficient to render the plant completely or partially resistant or tolerant to the herbicide (at a typical rate, unless otherwise specified; typical application rates can be found in the well-known *Herbicide Handbook* (Weed Science Society of America, Eighth Edition, 2002), for example). The herbicide can be applied at rates that would normally kill the target plant, at normal field use rates and concentrations. (Because of the subject invention, the level and/or concentration can optionally be higher than those that were previously used.) Preferably, plant cells and plants of the subject invention are protected against growth inhibition or injury caused by herbicide treatment. Transformed plants and plant cells of the subject invention are preferably rendered resistant or tolerant to an herbicide, as discussed herein, meaning that the transformed plant and plant cells can grow in the presence of effective amounts of one or more herbicides as discussed herein. Preferred proteins of the subject invention have catalytic activity to metabolize one or more aryloxyalkanoate compounds.

One cannot easily discuss the term “resistance” and not use the verb “tolerate” or the adjective “tolerant.” The industry has spent innumerable hours debating Herbicide Tolerant Crops (HTC) versus Herbicide Resistant Crops (HRC). HTC is a preferred term in the industry. However, the official Weed Science Society of America definition of resistance is “the inherited ability of a plant to survive and reproduce following exposure to a dose of herbicide normally lethal to the wild type. In a plant, resistance may be naturally occurring or induced by such techniques as genetic engineering or selection of variants produced by tissue culture or mutagenesis.” As used herein unless otherwise indicated, herbicide “resistance” is heritable and allows a plant to grow and reproduce in the presence of a typical herbicidally effective treatment by a herbicide for a given plant, as suggested by the current edition of *The Herbicide Handbook* as of the filing of the subject disclosure. As is recognized by those skilled in the art, a plant may still be considered “resistant” even though some degree of plant injury from herbicidal exposure is apparent. As used herein, the term “tolerance” is broader than the term “resistance,” and includes “resistance” as defined herein, as well as an improved capacity of a particular plant to withstand the various degrees of herbicidally induced injury that typically result in wild-type plants of the same genotype at the same herbicidal dose.

Transfer of the functional activity to plant or bacterial systems can involve a nucleic acid sequence, encoding the amino acid sequence for a protein of the subject invention, integrated into a protein expression vector appropriate to the host in which the vector will reside. One way to obtain a nucleic acid sequence encoding a protein with functional



US 11,371,055 B2

15

activity is to isolate the native genetic material from the bacterial species which produce the protein of interest, using information deduced from the protein's amino acid sequence, as disclosed herein. The native sequences can be optimized for expression in plants, for example, as discussed in more detail below. An optimized polynucleotide can also be designed based on the protein sequence.

The subject invention provides classes of proteins having novel activities as identified herein. One way to characterize these classes of proteins and the polynucleotides that encode them is by defining a polynucleotide by its ability to hybridize, under a range of specified conditions, with an exemplified nucleotide sequence (the complement thereof and/or a probe or probes derived from either strand) and/or by their ability to be amplified by PCR using primers derived from the exemplified sequences.

There are a number of methods for obtaining proteins for use according to the subject invention. For example, antibodies to the proteins disclosed herein can be used to identify and isolate other proteins from a mixture of proteins. Specifically, antibodies may be raised to the portions of the proteins that are most conserved or most distinct, as compared to other related proteins. These antibodies can then be used to specifically identify equivalent proteins with the characteristic activity by immunoprecipitation, enzyme linked immunosorbant assay (ELISA), or immuno-blotting. Antibodies to the proteins disclosed herein, or to equivalent proteins, or to fragments of these proteins, can be readily prepared using standard procedures. Such antibodies are an aspect of the subject invention. Antibodies of the subject invention include monoclonal and polyclonal antibodies, preferably produced in response to an exemplified or suggested protein.

One skilled in the art would readily recognize that proteins (and genes) of the subject invention can be obtained from a variety of sources. Since entire herbicide degradation operons are known to be encoded on transposable elements such as plasmids, as well as genomically integrated, proteins of the subject invention can be obtained from a wide variety of microorganisms, for example, including recombinant and/or wild-type bacteria.

Mutants of bacterial isolates can be made by procedures that are well known in the art. For example, asporogenous mutants can be obtained through ethylmethane sulfonate (EMS) mutagenesis of an isolate. The mutant strains can also be made using ultraviolet light and nitrosoguanidine by procedures well known in the art.

A protein "from" or "obtainable from" any of the subject isolates referred to or suggested herein means that the protein (or a similar protein) can be obtained from the isolate or some other source, such as another bacterial strain or a plant. "Derived from" also has this connotation, and includes proteins obtainable from a given type of bacterium that are modified for expression in a plant, for example. One skilled in the art will readily recognize that, given the disclosure of a bacterial gene and protein, a plant can be engineered to produce the protein. Antibody preparations, nucleic acid probes (DNA, RNA, or PNA, for example), and the like can be prepared using the polynucleotide and/or amino acid sequences disclosed herein and used to screen and recover other related genes from other (natural) sources.

Standard molecular biology techniques may be used to clone and sequence the proteins and genes described herein. Additional information may be found in Sambrook et al., 1989, which is incorporated herein by reference.

Polynucleotides and Probes. The subject invention further provides nucleic acid sequences that encode proteins for use

16

according to the subject invention. The subject invention further provides methods of identifying and characterizing genes that encode proteins having the desired herbicidal activity. In one embodiment, the subject invention provides unique nucleotide sequences that are useful as hybridization probes and/or primers for PCR techniques. The primers produce characteristic gene fragments that can be used in the identification, characterization, and/or isolation of specific genes of interest. The nucleotide sequences of the subject invention encode proteins that are distinct from previously described proteins.

The polynucleotides of the subject invention can be used to form complete "genes" to encode proteins or peptides in a desired host cell. For example, as the skilled artisan would readily recognize, the subject polynucleotides can be appropriately placed under the control of a promoter in a host of interest, as is readily known in the art. The level of gene expression and temporal/tissue specific expression can greatly impact the utility of the invention. Generally, greater levels of protein expression of a degradative gene will result in faster and more complete degradation of a substrate (in this case a target herbicide). Promoters will be desired to express the target gene at high levels unless the high expression has a consequential negative impact on the health of the plant. Typically, one would wish to have the AAD-12 gene constitutively expressed in all tissues for complete protection of the plant at all growth stages. However, one could alternatively use a vegetatively expressed resistance gene; this would allow use of the target herbicide in-crop for weed control and would subsequently control sexual reproduction of the target crop by application during the flowering stage. In addition, desired levels and times of expression can also depend on the type of plant and the level of tolerance desired. Some preferred embodiments use strong constitutive promoters combined with transcription enhancers and the like to increase expression levels and to enhance tolerance to desired levels. Some such applications are discussed in more detail below, before the Examples section.

As the skilled artisan knows, DNA typically exists in a double-stranded form. In this arrangement, one strand is complementary to the other strand and vice versa. As DNA is replicated in a plant (for example), additional complementary strands of DNA are produced. The "coding strand" is often used in the art to refer to the strand that binds with the anti-sense strand. The mRNA is transcribed from the "anti-sense" strand of DNA. The "sense" or "coding" strand has a series of codons (a codon is three nucleotides that can be read as a three-residue unit to specify a particular amino acid) that can be read as an open reading frame (ORF) to form a protein or peptide of interest. In order to produce a protein in vivo, a strand of DNA is typically transcribed into a complementary strand of mRNA which is used as the template for the protein. Thus, the subject invention includes the use of the exemplified polynucleotides shown in the attached sequence listing and/or equivalents including the complementary strands. RNA and PNA (peptide nucleic acids) that are functionally equivalent to the exemplified DNA molecules are included in the subject invention.

In one embodiment of the subject invention, bacterial isolates can be cultivated under conditions resulting in high multiplication of the microbe. After treating the microbe to provide single-stranded genomic nucleic acid, the DNA can be contacted with the primers of the invention and subjected to PCR amplification. Characteristic fragments of genes of interest will be amplified by the procedure, thus identifying the presence of the gene(s) of interest.

US 11,371,055 B2

17

Further aspects of the subject invention include genes and isolates identified using the methods and nucleotide sequences disclosed herein. The genes thus identified can encode herbicidal resistance proteins of the subject invention.

Proteins and genes for use according to the subject invention can be identified and obtained by using oligonucleotide probes, for example. These probes are detectable nucleotide sequences that can be detectable by virtue of an appropriate label or may be made inherently fluorescent as described in International Application No. WO 93/16094. The probes (and the polynucleotides of the subject invention) may be DNA, RNA, or PNA. In addition to adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U; for RNA molecules), synthetic probes (and polynucleotides) of the subject invention can also have inosine (a neutral base capable of pairing with all four bases; sometimes used in place of a mixture of all four bases in synthetic probes) and/or other synthetic (non-natural) bases. Thus, where a synthetic, degenerate oligonucleotide is referred to herein, and "N" or "n" is used generically, "N" or "n" can be G, A, T, C, or inosine. Ambiguity codes as used herein are in accordance with standard IUPAC naming conventions as of the filing of the subject application (for example, R means A or G, Y means C or T, etc.).

As is well known in the art, if a probe molecule hybridizes with a nucleic acid sample, it can be reasonably assumed that the probe and sample have substantial homology/similarity/identity. Preferably, hybridization of the polynucleotide is first conducted followed by washes under conditions of low, moderate, or high stringency by techniques well-known in the art, as described in, for example, Keller, G. H., M. M. Manak (1987) DNA Probes, Stockton Press, New York, N.Y., pp. 169-170. For example, as stated therein, low stringency conditions can be achieved by first washing with 2×SSC (Standard Saline Citrate)/0.1% SDS (Sodium Dodecyl Sulfate) for 15 minutes at room temperature. Two washes are typically performed. Higher stringency can then be achieved by lowering the salt concentration and/or by raising the temperature. For example, the wash described above can be followed by two washings with 0.1×SSC/0.1% SDS for 15 minutes each at room temperature followed by subsequent washes with 0.1×SSC/0.1% SDS for 30 minutes each at 55° C. These temperatures can be used with other hybridization and wash protocols set forth herein and as would be known to one skilled in the art (SSPE can be used as the salt instead of SSC, for example). The 2×SSC/0.1% SDS can be prepared by adding 50 ml of 20×SSC and 5 ml of 10% SDS to 445 ml of water. 20×SSC can be prepared by combining NaCl (175.3 g/0.150 M), sodium citrate (88.2 g/0.015 M), and water, adjusting pH to 7.0 with 10 N NaOH, then adjusting the volume to 1 liter. 10% SDS can be prepared by dissolving 10 g of SDS in 50 ml of autoclaved water, then diluting to 100 ml.

Detection of the probe provides a means for determining in a known manner whether hybridization has been maintained. Such a probe analysis provides a rapid method for identifying genes of the subject invention. The nucleotide segments used as probes according to the invention can be synthesized using a DNA synthesizer and standard procedures. These nucleotide sequences can also be used as PCR primers to amplify genes of the subject invention.

Hybridization characteristics of a molecule can be used to define polynucleotides of the subject invention. Thus the subject invention includes polynucleotides (and/or their complements, preferably their full complements) that hybridize with a polynucleotide exemplified herein. That is,

18

one way to define a gene (and the protein it encodes), for example, is by its ability to hybridize (under any of the conditions specifically disclosed herein) with a known or specifically exemplified gene.

As used herein, "stringent" conditions for hybridization refers to conditions which achieve the same, or about the same, degree of specificity of hybridization as the conditions employed by the current applicants. Specifically, hybridization of immobilized DNA on Southern blots with <sup>32</sup>P-labeled gene-specific probes can be performed by standard methods (see, e.g., Maniatis et al. 1982). In general, hybridization and subsequent washes can be carried out under conditions that allow for detection of target sequences. For double-stranded DNA gene probes, hybridization can be carried out overnight at 20-25° C. below the melting temperature (T<sub>m</sub>) of the DNA hybrid in 6×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature is described by the following formula (Beltz et al. 1983):

$$T_m = 81.5^\circ \text{C} + 16.6 \log [\text{Na}^+] + 0.41(\% \text{ G+C}) - 0.61(\% \text{ formamide}) - 600/\text{length of duplex in base pairs}.$$

Washes can typically be carried out as follows:

- (1) Twice at room temperature for 15 minutes in 1×SSPE, 0.1% SDS (low stringency wash).
- (2) Once at T<sub>m</sub>-20° C. for 15 minutes in 0.2×SSPE, 0.1% SDS (moderate stringency wash).

For oligonucleotide probes, hybridization can be carried out overnight at 10-20° C. below the melting temperature (T<sub>m</sub>) of the hybrid in 6×SSPE, 5×Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. T<sub>m</sub> for oligonucleotide probes can be determined by the following formula:

$$T_m(^{\circ} \text{C}) = 2(\text{number T/A base pairs}) + 4(\text{number G/C base pairs})$$

(Suggs et al., 1981).

Washes can typically be out as follows:

- (1) Twice at room temperature for 15 minutes 1×SSPE, 0.1% SDS (low stringency wash).
- (2) Once at the hybridization temperature for 15 minutes in 1×SSPE, 0.1% SDS (moderate stringency wash).

In general, salt and/or temperature can be altered to change stringency. With a labeled DNA fragment >70 or so bases in length, the following conditions can be used:

- Low: 1 or 2×SSPE, room temperature
- Low: 1 or 2×SSPE, 42° C.
- Moderate: 0.2× or 1×SSPE, 65° C.
- High: 0.1×SSPE, 65° C.

Duplex formation and stability depend on substantial complementarity between the two strands of a hybrid, and, as noted above, a certain degree of mismatch can be tolerated. Therefore, the probe sequences of the subject invention include mutations (both single and multiple), deletions, insertions of the described sequences, and combinations thereof, wherein said mutations, insertions and deletions permit formation of stable hybrids with the target polynucleotide of interest. Mutations, insertions, and deletions can be produced in a given polynucleotide sequence in many ways, and these methods are known to an ordinarily skilled artisan. Other methods may become known in the future.

PCR Technology. Polymerase Chain Reaction (PCR) is a repetitive, enzymatic, primed synthesis of a nucleic acid sequence. This procedure is well known and commonly used by those skilled in this art (see Mullis, U.S. Pat. Nos. 4,683,195, 4,683,202, and 4,800,159; Saiki et al., 1985). PCR is based on the enzymatic amplification of a DNA fragment of interest that is flanked by two oligonucleotide



US 11,371,055 B2

19

primers that hybridize to opposite strands of the target sequence. The primers are preferably oriented with the 3' ends pointing towards each other. Repeated cycles of heat denaturation of the template, annealing of the primers to their complementary sequences, and extension of the annealed primers with a DNA polymerase result in the amplification of the segment defined by the 5' ends of the PCR primers. The extension product of each primer can serve as a template for the other primer, so each cycle essentially doubles the amount of DNA fragment produced in the previous cycle. This results in the exponential accumulation of the specific target fragment, up to several million-fold in a few hours. By using a thermostable DNA polymerase such as Taq polymerase, isolated from the thermophilic bacterium *Thermus aquaticus*, the amplification process can be completely automated. Other enzymes which can be used are known to those skilled in the art.

Exemplified DNA sequences, or segments thereof, can be used as primers for PCR amplification. In performing PCR amplification, a certain degree of mismatch can be tolerated between primer and template. Therefore, mutations, deletions, and insertions (especially additions of nucleotides to the 5' end) of the exemplified primers fall within the scope of the subject invention. Mutations, insertions, and deletions can be produced in a given primer by methods known to an ordinarily skilled artisan.

Modification of genes and proteins. The subject genes and proteins can be fused to other genes and proteins to produce chimeric or fusion proteins. The genes and proteins useful according to the subject invention include not only the specifically exemplified full-length sequences, but also portions, segments and/or fragments (including contiguous fragments and internal and/or terminal deletions compared to the full-length molecules) of these sequences, variants, mutants, chimerics, and fusions thereof. Proteins of the subject invention can have substituted amino acids so long as they retain desired functional activity. "Variant" genes have nucleotide sequences that encode the same proteins or equivalent proteins having activity equivalent or similar to an exemplified protein.

The top two results of BLAST searches with the native aad-12 nucleotide sequence show a reasonable level of homology (about 85%) over 120 base pairs of sequence. Hybridization under certain conditions could be expected to include these two sequences. See GENBANK Acc. Nos. DQ406818.1 (89329742; *Rhodospirillum rubrum*) and AJ6288601.1 (44903451; *Sphingomonas*). *Rhodospirillum rubrum* is very similar to *Delftia* but *Sphingomonas* is an entirely different Class phylogenetically.

The terms "variant proteins" and "equivalent proteins" refer to proteins having the same or essentially the same biological/functional activity against the target substrates and equivalent sequences as the exemplified proteins. As used herein, reference to an "equivalent" sequence refers to sequences having amino acid substitutions, deletions, additions, or insertions that improve or do not adversely affect activity to a significant extent. Fragments retaining activity are also included in this definition. Fragments and other equivalents that retain the same or similar function or activity as a corresponding fragment of an exemplified protein are within the scope of the subject invention. Changes, such as amino acid substitutions or additions, can be made for a variety of purposes, such as increasing (or decreasing) protease stability of the protein (without materially/substantially decreasing the functional activity of the protein), removing or adding a restriction site, and the like.

20

Variations of genes may be readily constructed using standard techniques for making point mutations, for example.

In addition, U.S. Pat. No. 5,605,793, for example, describes methods for generating additional molecular diversity by using DNA reassembly after random or focused fragmentation. This can be referred to as gene "shuffling," which typically involves mixing fragments (of a desired size) of two or more different DNA molecules, followed by repeated rounds of renaturation. This can improve the activity of a protein encoded by a starting gene. The result is a chimeric protein having improved activity, altered substrate specificity, increased enzyme stability, altered stereospecificity, or other characteristics.

"Shuffling" can be designed and targeted after obtaining and examining the atomic 3D (three dimensional) coordinates and crystal structure of a protein of interest. Thus, "focused shuffling" can be directed to certain segments of a protein that are ideal for modification, such as surface-exposed segments, and preferably not internal segments that are involved with protein folding and essential 3D structural integrity.

Specific changes to the "active site" of the enzyme can be made to affect the inherent functionality with respect to activity or stereospecificity (see alignment FIG. 2). Muller et al. (2006). The known tauD crystal structure was used as a model dioxygenase to determine active site residues while bound to its inherent substrate taurine. Elkins et al. (2002) "X-ray crystal structure of *Escherichia coli* taurine/alpha-ketoglutarate dioxygenase complexed to ferrous iron and substrates," *Biochemistry* 41(16):5185-5192. Regarding sequence optimization and designability of enzyme active sites, see Chakrabarti et al., PNAS, (Aug. 23, 2005), 102 (34):12035-12040.

Variant genes can be used to produce variant proteins; recombinant hosts can be used to produce the variant proteins. Using these "gene shuffling" techniques, equivalent genes and proteins can be constructed that comprise any 5, 10, or 20 contiguous residues (amino acid or nucleotide) of any sequence exemplified herein. As one skilled in the art knows, the gene shuffling techniques, for example, can be adjusted to obtain equivalents having, for example, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106, 107, 108, 109, 110, 111, 112, 113, 114, 115, 116, 117, 118, 119, 120, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139, 140, 141, 142, 143, 144, 145, 146, 147, 148, 149, 150, 151, 152, 153, 154, 155, 156, 157, 158, 159, 160, 161, 162, 163, 164, 165, 166, 167, 168, 169, 170, 171, 172, 173, 174, 175, 176, 177, 178, 179, 180, 181, 182, 183, 184, 185, 186, 187, 188, 189, 190, 191, 192, 193, 194, 195, 196, 197, 198, 199, 200, 201, 202, 203, 204, 205, 206, 207, 208, 209, 210, 211, 212, 213, 214, 215, 216, 217, 218, 219, 220, 221, 222, 223, 224, 225, 226, 227, 228, 229, 230, 231, 232, 233, 234, 235, 236, 237, 238, 239, 240, 241, 242, 243, 244, 245, 246, 247, 248, 249, 250, 251, 252, 253, 254, 255, 256, 257, 258, 259, 260, 261, 262, 263, 264, 265, 266, 267, 268, 269, 270, 271, 272, 273, 274, 275, 276, 277, 278, 279, 280, 281, 282, 283, 284, 285, 286, 287, 288, 289, 290, 291, 292, or 293 contiguous residues (amino acid or nucleotide), corresponding to a segment (of the same size) in any of the exemplified or suggested sequences (or the complements (full comple-

US 11,371,055 B2

21

ments) thereof). Similarly sized segments, especially those for conserved regions, can also be used as probes and/or primers.

Fragments of full-length genes can be made using commercially available exonucleases or endonucleases according to standard procedures. For example, enzymes such as Bal31 or site-directed mutagenesis can be used to systematically cut off nucleotides from the ends of these genes. Also, genes that encode active fragments may be obtained using a variety of restriction enzymes. Proteases may be used to directly obtain active fragments of these proteins.

It is within the scope of the invention as disclosed herein that proteins can be truncated and still retain functional activity. By "truncated protein" it is meant that a portion of a protein may be cleaved off while the remaining truncated protein retains and exhibits the desired activity after cleavage. Cleavage can be achieved by various proteases. Furthermore, effectively cleaved proteins can be produced using molecular biology techniques wherein the DNA bases encoding said protein are removed either through digestion with restriction endonucleases or other techniques available to the skilled artisan. After truncation, said proteins can be expressed in heterologous systems such as *E. coli*, baculoviruses, plant-based viral systems, yeast, and the like and then placed in insect assays as disclosed herein to determine activity. It is well-known in the art that truncated proteins can be successfully produced so that they retain functional activity while having less than the entire, full-length sequence. For example, B.t. proteins can be used in a truncated (core protein) form (see, e.g., Höfte et al. (1989), and Adang et al. (1985)). As used herein, the term "protein" can include functionally active truncations.

In some cases, especially for expression in plants, it can be advantageous to use truncated genes that express truncated proteins. Preferred truncated genes will typically encode 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% of the full-length protein.

Certain proteins of the subject invention have been specifically exemplified herein. As these proteins are merely exemplary of the proteins of the subject invention, it should be readily apparent that the subject invention comprises variant or equivalent proteins (and nucleotide sequences coding for equivalents thereof) having the same or similar activity of the exemplified proteins. Equivalent proteins will have amino acid similarity (and/or homology) with an exemplified protein. The amino acid identity will typically be at least 60%, preferably at least 75%, more preferably at least 80%, even more preferably at least 90%, and can be at least 95%. Preferred proteins of the subject invention can also be defined in terms of more particular identity and/or similarity ranges. For example, the identity and/or similarity can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified or suggested herein. Any number listed above can be used to define the upper and lower limits.

Unless otherwise specified, as used herein, percent sequence identity and/or similarity of two nucleic acids is determined using the algorithm of Karlin and Altschul, 1990, modified as in Karlin and Altschul 1993. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al., 1990. BLAST nucleotide searches are performed with the NBLAST program,

22

score=100, wordlength=12. Gapped BLAST can be used as described in Altschul et al., 1997. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) are used. See NCBI/NIH website. To obtain gapped alignments for comparison purposes, the AlignX function of Vector NTI Suite 8 (InforMax, Inc., North Bethesda, Md., U.S.A.), was used employing the default parameters. These were: a Gap opening penalty of 15, a Gap extension penalty of 6.66, and a Gap separation penalty range of 8.

Various properties and three-dimensional features of the protein can also be changed without adversely affecting the activity/functionality of the protein. Conservative amino acid substitutions can be tolerated/made to not adversely affect the activity and/or three-dimensional configuration of the molecule. Amino acids can be placed in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby an amino acid of one class is replaced with another amino acid of the same type fall within the scope of the subject invention so long as the substitution is not adverse to the biological activity of the compound. Table 2 provides a listing of examples of amino acids belonging to each class.

TABLE 2

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

In some instances, non-conservative substitutions can also be made. However, preferred substitutions do not significantly detract from the functional/biological activity of the protein.

As used herein, reference to "isolated" polynucleotides and/or "purified" proteins refers to these molecules when they are not associated with the other molecules with which they would be found in nature. Thus, reference to "isolated" and/or "purified" signifies the involvement of the "hand of man" as described herein. For example, a bacterial "gene" of the subject invention put into a plant for expression is an "isolated polynucleotide." Likewise, a protein derived from a bacterial protein and produced by a plant is an "isolated protein."

Because of the degeneracy/redundancy of the genetic code, a variety of different DNA sequences can encode the amino acid sequences disclosed herein. It is well within the skill of a person trained in the art to create alternative DNA sequences that encode the same, or essentially the same, proteins. These variant DNA sequences are within the scope of the subject invention. This is also discussed in more detail below in the section entitled "Optimization of sequence for expression in plants."

Optimization of Sequence for Expression in Plants. To obtain high expression of heterologous genes in plants it is generally preferred to reengineer the genes so that they are more efficiently expressed in (the cytoplasm of) plant cells. Maize is one such plant where it may be preferred to re-design the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression, using codon bias more closely aligned with the target plant sequence, whether a dicot or monocot species.

US 11,371,055 B2

23

Sequences can also be optimized for expression in any of the more particular types of plants discussed elsewhere herein.

Transgenic Hosts. The protein-encoding genes of the subject invention can be introduced into a wide variety of microbial or plant hosts. The subject invention includes transgenic plant cells and transgenic plants. Preferred plants (and plant cells) are corn, *Arabidopsis*, tobacco, soybeans, cotton, canola, rice, wheat, turf, legume forages (e.g., alfalfa and clover), pasture grasses, and the like. Other types of transgenic plants can also be made according to the subject invention, such as fruits, vegetables, ornamental plants, and trees. More generally, dicots and/or monocots can be used in various aspects of the subject invention.

In preferred embodiments, expression of the gene results, directly or indirectly, in the intracellular production (and maintenance) of the protein(s) of interest. Plants can be rendered herbicide-resistant in this manner. Such hosts can be referred to as transgenic, recombinant, transformed, and/or transfected hosts and/or cells. In some aspects of this invention (when cloning and preparing the gene of interest, for example), microbial (preferably bacterial) cells can be produced and used according to standard techniques, with the benefit of the subject disclosure.

Plant cells transfected with a polynucleotide of the subject invention can be regenerated into whole plants. The subject invention includes cell cultures including tissue cell cultures, liquid cultures, and plated cultures. Seeds produced by and/or used to generate plants of the subject invention are also included within the scope of the subject invention. Other plant tissues and parts are also included in the subject invention. The subject invention likewise includes methods of producing plants or cells comprising a polynucleotide of the subject invention. One preferred method of producing such plants is by planting a seed of the subject invention.

Although plants can be preferred, the subject invention also includes production of highly active recombinant AAD-12 in a *Pseudomonas fluorescens* (Pf) host strain, for example. The subject invention includes preferred growth temperatures for maintaining soluble active AAD-12 in this host; a fermentation condition where AAD-12 is produced as more than 40% total cell protein, or at least 10 g/L; a purification process results high recovery of active recombinant AAD-12 from a Pf host; a purification scheme which yields at least 10 g active AAD-12 per kg of cells; a purification scheme which can yield 20 g active AAD-12 per kg of cells; a formulation process that can store and restore AAD-12 activity in solution; and a lyophilization process that can retain AAD-12 activity for long-term storage and shelf life.

Insertion of Genes to Form Transgenic Hosts. One aspect of the subject invention is the transformation/transfection of plants, plant cells, and other host cells with polynucleotides of the subject invention that express proteins of the subject invention. Plants transformed in this manner can be rendered resistant to a variety of herbicides with different modes of action.

A wide variety of methods are available for introducing a gene encoding a desired protein into the target host under conditions that allow for stable maintenance and expression of the gene. These methods are well known to those skilled in the art and are described, for example, in U.S. Pat. No. 5,135,867.

Vectors comprising an AAD-12 polynucleotide are included in the scope of the subject invention. For example, a large number of cloning vectors comprising a replication system in *E. coli* and a marker that permits selection of the transformed cells are available for preparation for the inser-

24

tion of foreign genes into higher plants. The vectors comprise, for example, pBR322, pUC series, M13mp series, pACYC184, etc. Accordingly, the sequence encoding the protein can be inserted into the vector at a suitable restriction site. The resulting plasmid is used for transformation into *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium, then harvested and lysed. The plasmid is recovered by purification away from genomic DNA. Sequence analysis, restriction analysis, electrophoresis, and other biochemical-molecular biological methods are generally carried out as methods of analysis. After each manipulation, the DNA sequence used can be restriction digested and joined to the next DNA sequence. Each plasmid sequence can be cloned in the same or other plasmids. Depending on the method of inserting desired genes into the plant, other DNA sequences may be necessary. If, for example, the Ti or Ri plasmid is used for the transformation of the plant cell, then at least the right border, but often the right and the left border of the Ti or Ri plasmid T-DNA, has to be joined as the flanking region of the genes to be inserted. The use of T-DNA for the transformation of plant cells has been intensively researched and described in EP 120 516; Hoekema (1985); Fraley et al. (1986); and An et al. (1985).

A large number of techniques are available for inserting DNA into a plant host cell. Those techniques include transformation with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as transformation agent, fusion, injection, biolistics (microparticle bombardment), silicon carbide whiskers, aerosol beaming, PEG, or electroporation as well as other possible methods. If *Agrobacteria* are used for the transformation, the DNA to be inserted has to be cloned into special plasmids, namely either into an intermediate vector or into a binary vector. The intermediate vectors can be integrated into the Ti or Ri plasmid by homologous recombination owing to sequences that are homologous to sequences in the T-DNA. The Ti or Ri plasmid also comprises the vir region necessary for the transfer of the T-DNA. Intermediate vectors cannot replicate themselves in *Agrobacteria*. The intermediate vector can be transferred into *Agrobacterium tumefaciens* by means of a helper plasmid (conjugation). Binary vectors can replicate themselves both in *E. coli* and in *Agrobacteria*. They comprise a selection marker gene and a linker or polylinker which are framed by the right and left T-DNA border regions. They can be transformed directly into *Agrobacteria* (Holsters, 1978). The *Agrobacterium* used as host cell is to comprise a plasmid carrying a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be contained. The bacterium so transformed is used for the transformation of plant cells. Plant explants can be cultivated advantageously with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* for the transfer of the DNA into the plant cell. Whole plants can then be regenerated from the infected plant material (for example, pieces of leaf, segments of stalk, roots, but also protoplasts or suspension-cultivated cells) in a suitable medium, which may contain antibiotics or biocides for selection. The plants so obtained can then be tested for the presence of the inserted DNA. No special demands are made of the plasmids in the case of injection and electroporation. It is possible to use ordinary plasmids, such as, for example, pUC derivatives.

The transformed cells grow inside the plants in the usual manner. They can form germ cells and transmit the transformed trait(s) to progeny plants. Such plants can be grown in the normal manner and crossed with plants that have the same transformed hereditary factors or other hereditary



US 11,371,055 B2

25

factors. The resulting hybrid individuals have the corresponding phenotypic properties.

In some preferred embodiments of the invention, genes encoding the bacterial protein are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and enable selection of transformed plant lines expressing mRNA encoding the proteins.

Once the inserted DNA has been integrated in the genome, it is relatively stable there (and does not come out again). It normally contains a selection marker that confers on the transformed plant cells resistance to a biocide or an antibiotic, such as kanamycin, G418, bleomycin, hygromycin, or chloramphenicol, inter alia. Plant selectable markers also typically can provide resistance to various herbicides such as glufosinate (e.g., PAT/bar), glyphosate (EPSPS), ALS-inhibitors (e.g., imidazolinone, sulfonyleurea, triazolo-pyrimidine sulfonamide, et al.), bromoxynil, HPPD-inhibitor resistance, PPO-inhibitors, ACC-ase inhibitors, and many others. The individually employed marker should accordingly permit the selection of transformed cells rather than cells that do not contain the inserted DNA. The gene(s) of interest are preferably expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated into proteins, thereby incorporating amino acids of interest into protein. The genes encoding a protein expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter, or an inducible promoter.

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include the introduction of genetic material coated onto microparticles directly into cells (U.S. Pat. No. 4,945,050 to Cornell and U.S. Pat. No. 5,141,131 to Dow-Elanco, now Dow AgroSciences, LLC). In addition, plants may be transformed using *Agrobacterium* technology, see U.S. Pat. No. 5,177,010 to University of Toledo; U.S. Pat. No. 5,104,310 to Texas A&M; European Patent Application 0131624B1; European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot; U.S. Pat. Nos. 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot; European Patent Applications 116718, 290799, 320500, all to Max Planck; European Patent Applications 604662 and 627752, and U.S. Pat. No. 5,591,616, to Japan Tobacco; European Patent Applications 0267159 and 0292435, and U.S. Pat. No. 5,231,019, all to Ciba Geigy, now Syngenta; U.S. Pat. Nos. 5,463,174 and 4,762,785, both to Calgene; and U.S. Pat. Nos. 5,004,863 and 5,159,135, both to Agracetus. Other transformation technology includes whiskers technology. See U.S. Pat. Nos. 5,302,523 and 5,464,765, both to Zeneca, now Syngenta. Other direct DNA delivery transformation technology includes aerosol beam technology. See U.S. Pat. No. 6,809,232. Electroporation technology has also been used to transform plants. See WO 87/06614 to Boyce Thompson Institute; U.S. Pat. Nos. 5,472,869 and 5,384,253, both to Dekalb; and WO 92/09696 and WO 93/21335, both to Plant Genetic Systems. Furthermore, viral vectors can also be used to produce transgenic plants expressing the protein of interest. For example, monocotyledonous plants can be transformed with a viral vector using the methods described in U.S. Pat. No. 5,569,597 to Mycogen Plant Science and Ciba-Geigy (now Syngenta), as well as U.S. Pat. Nos. 5,589,367 and 5,316,931, both to Biosource, now Large Scale Biology.

26

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method that provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform *Agrobacterium* mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation. Where *Agrobacterium* is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the *Agrobacterium* host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

In some cases where *Agrobacterium* is used for transformation, the expression construct being within the T-DNA borders will be inserted into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al. (1980) and EPO 0 120 515. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed *Agrobacterium* and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using *Agrobacterium*, explants may be combined and incubated with the transformed *Agrobacterium* for sufficient time to allow transformation thereof. After transformation, the *Agrobacteria* are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encouraged by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations. Regardless of transformation technique, the gene encoding a bacterial protein is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue that is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, root tissue, tissues for expression in phloem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

As mentioned above, a variety of selectable markers can be used, if desired. Preference for a particular marker is at

US 11,371,055 B2

27

the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G41, hygromycin resistance; methotrexate resistance, as well as those genes which encode for resistance or tolerance to glyphosate; phosphinothricin (bialaphos or glufosinate); ALS-inhibiting herbicides (imidazolinones, sulfonylureas and triazopyrimidine herbicides), ACC-ase inhibitors (e.g., aryloxypropionates or cyclohexanediones), and others such as bromoxynil, and HPPD-inhibitors (e.g., mesotrione) and the like.

In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes that are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in Weising et al., 1988. Preferred reporter genes include the beta-glucuronidase (GUS) of the uidA locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the uidA locus of *E. coli* as described by Jefferson et al., (1987) to identify transformed cells.

In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, see U.S. Pat. No. 6,166,302, especially Example 7E) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter. ADH promoter, heat-shock promoters, and tissue specific promoters. Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, osmotin UTR sequences, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan. Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific

28

cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

Promoter regulatory elements may also be active (or inactive) during a certain stage of the plant's development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo-specific, corn-silk-specific, cotton-fiber-specific, root-specific, seed-endosperm-specific, or vegetative phase-specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes), light (RUBP carboxylase), hormone (Em), metabolites, chemical (tetracycline responsive), and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

Plant RNA viral based systems can also be used to express bacterial protein. In so doing, the gene encoding a protein can be inserted into the coat promoter region of a suitable plant virus which will infect the host plant of interest. The protein can then be expressed thus providing protection of the plant from herbicide damage. Plant RNA viral based systems are described in U.S. Pat. No. 5,500,360 to Mycogen Plant Sciences, Inc. and U.S. Pat. Nos. 5,316,931 and 5,589,367 to Biosource, now Large Scale Biology.

Means of Further Increasing Tolerance or Resistance Levels. It is shown herein that plants of the subject invention can be imparted with novel herbicide resistance traits without observable adverse effects on phenotype including yield. Such plants are within the scope of the subject invention. Plants exemplified and suggested herein can withstand 2x, 3x, 4x, and 5x typical application levels, for example, of at least one subject herbicide. Improvements in these tolerance levels are within the scope of this invention. For example, various techniques are known in the art, and can foreseeably be optimized and further developed, for increasing expression of a given gene.

One such method includes increasing the copy number of the subject AAD-12 genes (in expression cassettes and the like). Transformation events can also be selected for those having multiple copies of the genes.

Strong promoters and enhancers can be used to "supercharge" expression. Examples of such promoters include the preferred 35T promoter which uses 35S enhancers. 35S, maize ubiquitin, *Arabidopsis* ubiquitin, A.t. actin, and CSMV promoters are included for such uses. Other strong viral promoters are also preferred. Enhancers include 4 OCS and the 35S double enhancer. Matrix attachment regions (MARs) can also be used to increase transformation efficiencies and transgene expression, for example.

Shuffling (directed evolution) and transcription factors can also be used for embodiments according to the subject invention.

Variant proteins can also be designed that differ at the sequence level but that retain the same or similar overall essential three-dimensional structure, surface charge distribution, and the like. See e.g. U.S. Pat. No. 7,058,515; Larson et al., Protein Sci. 2002 11: 2804-2813, "Thoroughly sampling sequence space: Large-scale protein design of structural ensembles."; Crameri et al., *Nature Biotechnology* 15, 436-438 (1997), "Molecular evolution of an arsenate detoxification pathway by DNA shuffling."; Stemmer, W. P. C. 1994. DNA shuffling by random fragmentation and reassembly: in vitro recombination for molecular evolution.

US 11,371,055 B2

29

Proc. Natl. Acad. Sci. USA 91: 10747-10751; Stemmer, W. P. C. 1994. Rapid evolution of a protein in vitro by DNA shuffling. *Nature* 370: 389-391; Stemmer, W. P. C. 1995. Searching sequence space. *Bio/Technology* 13: 549-553; Cramer, A., Cwirla, S., and Stemmer, W. P. C. 1996. Construction and evolution of antibody-phage libraries by DNA shuffling. *Nature Medicine* 2: 100-103; and Cramer, A., Whitehorn, E. A., Tate, E. and Stemmer, W. P. C. 1996. Improved green fluorescent protein by molecular evolution using DNA shuffling. *Nature Biotechnology* 14: 315-319.

The activity of recombinant polynucleotides inserted into plant cells can be dependent upon the influence of endogenous plant DNA adjacent the insert. Thus, another option is taking advantage of events that are known to be excellent locations in a plant genome for insertions. See e.g. WO 2005/103266 A1, relating to cry1F and cry1Ac cotton events; the subject AAD-12 gene can be substituted in those genomic loci in place of the cry1F and/or cry1Ac inserts. Thus, targeted homologous recombination, for example, can be used according to the subject invention. This type of technology is the subject of, for example, WO 03/080809 A2 and the corresponding published U.S. application (USPA 20030232410), relating to the use of zinc fingers for targeted recombination. The use of recombinases (cre-lox and flp-frt for example) is also known in the art.

AAD-12 detoxification is believed to occur in the cytoplasm. Thus, means for further stabilizing this protein and mRNAs (including blocking mRNA degradation) are included in aspects of the subject invention, and art-known techniques can be applied accordingly. The subject proteins can be designed to resist degradation by proteases and the like (protease cleavage sites can be effectively removed by re-engineering the amino acid sequence of the protein). Such embodiments include the use of 5' and 3' stem loop structures like UTRs from osmotin, and per5 (AU-rich untranslated 5' sequences). 5' caps like 7-methyl or 2'-O-methyl groups, e.g., 7-methylguanylic acid residue, can also be used. See, e.g., Proc. Natl. Acad. Sci. USA Vol. 74, No. 7, pp. 2734-2738 (July 1977) *Importance of 5'-terminal blocking structure to stabilize mRNA in eukaryotic protein synthesis*. Protein complexes or ligand blocking groups can also be used.

Computational design of 5' or 3' UTR most suitable for AAD-12 (synthetic hairpins) can also be conducted within the scope of the subject invention. Computer modeling in general, as well as gene shuffling and directed evolution, are discussed elsewhere herein. More specifically regarding computer modeling and UTRs, computer modeling techniques for use in predicting/evaluating 5' and 3' UTR derivatives of the present invention include, but are not limited to: MFold version 3.1 available from Genetics Corporation Group, Madison, Wis. (see Zucker et al., Algorithms and Thermodynamics for RNA Secondary Structure Prediction: A Practical Guide. In *RNA Biochemistry and Biotechnology*, 11-43, J. Barciszewski & B. F. C. Clark, eds., NATO ASI Series, Kluwer Academic Publishers, Dordrecht, NL, (1999); Zucker et al., *Expanded Sequence Dependence of Thermodynamic Parameters Improves Prediction of RNA Secondary Structure*. *J. Mol. Biol.* 288, 911-940 (1999); Zucker et al., RNA Secondary Structure Prediction. In *Current Protocols in Nucleic Acid Chemistry* S. Beaucage, D. E. Bergstrom, G. D. Glick, and R. A. Jones eds., John Wiley & Sons, New York, 11.2.1-11.2.10, (2000)), COVE (RNA structure analysis using covariance models (stochastic context free grammar methods)) v.2.4.2 (Eddy & Durbin, Nucl. Acids Res. 1994, 22: 2079-2088) which is freely distributed as source code and which can be downloaded by

30

accessing the website [genetics.wustl.edu/eddysoftware/](http://genetics.wustl.edu/eddysoftware/), and FOLDALIGN, also freely distributed and available for downloading at the website [bioinf.au.dk](http://bioinf.au.dk). FOLDALIGN/ (see *Finding the most significant common sequence and structure motifs in a set of RNA sequences*. J. Gorodkin, L. J. Heyer and G. D. Stormo. *Nucleic Acids Research*. Vol. 25, no. 18 pp 3724-3732, 1997; *Finding Common Sequence and Structure Motifs in a set of RNA Sequences*. J. Gorodkin, L. J. Heyer, and G. D. Stormo. *ISMB* 5; 120-123, 1997).

Embodiments of the subject invention can be used in conjunction with naturally evolved or chemically induced mutants (mutants can be selected by screening techniques, then transformed with AAD-12 and possibly other genes). Plants of the subject invention can be combined with ALS resistance and/or evolved glyphosate resistance. Aminopyralid resistance, for example, can also be combined or "stacked" with an AAD-12 gene.

Traditional breeding techniques can also be combined with the subject invention to powerfully combine, introgress, and improve desired traits.

Further improvements also include use with appropriate safeners to further protect plants and/or to add cross resistance to more herbicides. (Safeners typically act to increase plants immune system by activating/expressing cP450. Safeners are chemical agents that reduce the phytotoxicity of herbicides to crop plants by a physiological or molecular mechanism, without compromising weed control efficacy.)

Herbicide safeners include benoxacor, cloquintocet, cyometrinil, dichlormid, dicyclonon, dietholate, fenclorazole, fenclorim, flurazole, fluxofenim, furilazole, isoxadifen, mefenpyr, mephenate, naphthalic anhydride, and oxab-trinil. Plant activators (a new class of compounds that protect plants by activating their defense mechanisms) can also be used in embodiments of the subject invention. These include acibenzolar and probenazole.

Commercialized safeners can be used for the protection of large-seeded grass crops, such as corn, grain *sorghum*, and wet-sown rice, against preplant-incorporated or preemergence-applied herbicides of the thiocarbamate and chloroacetanilide families. Safeners also have been developed to protect winter cereal crops such as wheat against postemergence applications of aryloxyphenoxypropionate and sulfonylurea herbicides. The use of safeners for the protection of corn and rice against sulfonylurea, imidazolinone, cyclohexanedione, isoxazole, and triketone herbicides is also well-established. A safener-induced enhancement of herbicide detoxification in safened plants is widely accepted as the major mechanism involved in safener action. Safeners induce cofactors such as glutathione and herbicide-detoxifying enzymes such as glutathione S-transferases, cytochrome P450 monooxygenases, and glucosyl transferases. Hatzios K K, Burgos N (2004) "Metabolism-based herbicide resistance: regulation by safeners," *Weed Science*: Vol. 52, No. 3 pp. 454-467.

Use of a cytochrome p450 monooxygenase gene stacked with AAD-12 is one preferred embodiment. There are P450s involved in herbicide metabolism; cP450 can be of mammalian or plant origin, for example. In higher plants, cytochrome P450 monooxygenase (P450) is known to conduct secondary metabolism. It also plays an important role in the oxidative metabolism of xenobiotics in cooperation with NADPH-cytochrome P450 oxidoreductase (reductase). Resistance to some herbicides has been reported as a result of the metabolism by P450 as well as glutathione S-transferase. A number of microsomal P450 species involved in xenobiotic metabolism in mammals have been characterized by molecular cloning. Some of them were reported to



US 11,371,055 B2

31

metabolize several herbicides efficiently. Thus, transgenic plants with plant or mammalian P450 can show resistance to several herbicides.

One preferred embodiment of the foregoing is the use of cP450 for resistance to acetochlor (acetochlor-based products include Surpass®, Keystone®, Keystone LA, Ful-Time® and TopNotch® herbicides) and/or trifluralin (such as Treflan®). Such resistance in soybeans and/or corn is included in some preferred embodiments. For additional guidance regarding such embodiments, see e.g. Inui et al., "A selectable marker using cytochrome P450 monooxygenases for *Arabidopsis* transformation," *Plant Biotechnology* 22, 281-286 (2005) (relating to a selection system for transformation of *Arabidopsis thaliana* via *Agrobacterium tumefaciens* that uses human cytochrome P450 monooxygenases that metabolize herbicides; herbicide tolerant seedlings were transformed and selected with the herbicides acetochlor, amiprofos-methyl, chlorpropham, chlorsulfuron, norflurazon, and pendimethalin); Siminszky et al., "Expression of a soybean cytochrome P450 monooxygenase cDNA in yeast and tobacco enhances the metabolism of phenylurea herbicides," *PNAS* Vol. 96, Issue 4, 1750-1755, Feb. 16, 1999; Sheldon et al, *Weed Science*: Vol. 48, No. 3, pp. 291-295, "A cytochrome P450 monooxygenase cDNA (CYP71A10) confers resistance to linuron in transgenic *Nicotiana tabacum*"; and "Phytoremediation of the herbicides atrazine and metolachlor by transgenic rice plants expressing human CYP1A1, CYP2B6, and CYP2C19," *J Agric Food Chem.* 2006 Apr. 19; 54(8):2985-91 (relating to testing a human cytochrome p450 monooxygenase in rice where the rice plants reportedly showed high tolerance to chloroacetamides (acetochlor, alachlor, metolachlor, pretilachlor, and thenylchlor), oxyacetamides (mefenacet), pyridazinones (norflurazon), 2,6-dinitroanilines (trifluralin and pendimethalin), phosphamides (amiprofos-methyl, thiocarbamates (pyributicarb), and ureas (chlortoluron)).

There is also the possibility of altering or using different 2,4-D chemistries to make the subject AAD-12 genes more efficient. Such possible changes include creating better substrates and better leaving groups (higher electronegativity).

Auxin transport inhibitors (e.g. diflufenzopyr) can also be used to increase herbicide activity with 2,4-D.

Unless specifically indicated or implied, the terms "a", "an", and "the" signify "at least one" as used herein.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

Following are examples that illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

#### Example 1—Method for Identifying Genes that Impart Resistance to 2,4-D in Planta

As a way to identify genes which possess herbicide degrading activities in planta, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (i.e.,  $\alpha$ -ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul et al., 1997) algorithm to compare against available NCBI protein sequences

32

deposited. Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level. Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with  $\geq 50\%$  homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation (relative to tfdA from *Ralstonia eutropha*) can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

A single gene (sdpA) was identified from the NCBI database (see the [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov) website; accession #AF516752) as a homologue with only 31% amino acid identity to tfdA. Percent identity was determined by first translating both the sdpA and tfdA DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

#### Example 2—Optimization of Sequence for Expression in Plants and Bacteria

##### 2.1—Background.

To obtain higher levels of expression of heterologous genes in plants, it may be preferred to reengineer the protein encoding sequence of the genes so that they are more efficiently expressed in plant cells. Maize is one such plant where it may be preferred to re-design the heterologous protein coding region prior to transformation to increase the expression level of the gene and the level of encoded protein in the plant. Therefore, an additional step in the design of genes encoding a bacterial protein is reengineering of a heterologous gene for optimal expression.

One reason for the reengineering of a bacterial protein for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding a bacterial protein for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding a bacterial protein is to generate a DNA sequence in which the sequence modifications do not hinder translation.

Table 3 illustrates how high the G+C content is in maize. For the data in Table 3, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (Accelrys, San Diego, Calif.). Intron sequences were ignored in the calculations.

US 11,371,055 B2

33

TABLE 3

Compilation of G + C contents of protein coding regions of maize genes		
Protein Class <sup>a</sup>	Range % G + C	Mean % G + C <sup>b</sup>
Metabolic Enzymes (76)	44.4-75.3	59.0 (+-.8.0)
Structural Proteins (18)	48.6-70.5	63.6 (+-.6.7)
Regulatory Proteins (5)	57.2-68.8	62.0 (+-.4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (+-.7.2)
All Proteins (108)	44.4-75.3	60.8 (+-.5.2) <sup>c</sup>

<sup>a</sup>Number of genes in class given in parentheses.<sup>b</sup>Standard deviations given in parentheses.<sup>c</sup>Combined groups mean ignored in mean calculation

Due to the plasticity afforded by the redundancy/degeneracy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This “codon bias” is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons, whereas those having higher G+C contents utilize codons having G or C in the third position. It is thought that the presence of “minor” codons within an mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

In engineering genes encoding a bacterial protein for maize (or other plant, such as cotton or soybean) expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 4. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary codons preferred by the plant should be determined, as well as the second, third, and fourth choices of preferred codons when multiple choices exist. A new DNA sequence can then be designed which encodes the amino sequence of the bacterial protein, but the new DNA sequence differs from the native bacterial DNA sequence (encoding the protein) by the substitution of the plant (first preferred, second preferred, third preferred, or fourth preferred) codons to specify the amino acid at each position within the protein amino acid sequence. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with first, second, third, or fourth choice preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron junctions (5' or 3'), poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

34

TABLE 4

Preferred amino acid codons for proteins expressed in maize	
Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tyrosine	TAC/TAT
Stop	TGA/TAG

It is preferred that the plant optimized gene(s) encoding a bacterial protein contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third or fourth choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice codons, wherein the total percentage is 100%. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in PCT application WO 97/13402.

Thus, in order to design plant optimized genes encoding a bacterial protein, a DNA sequence is designed to encode the amino acid sequence of said protein utilizing a redundant genetic code established from a codon bias table compiled from the gene sequences for the particular plant or plants. The resulting DNA sequence has a higher degree of codon diversity, a desirable base composition, can contain strategically placed restriction enzyme recognition sites, and lacks sequences that might interfere with transcription of the gene, or translation of the product mRNA. Thus, synthetic genes that are functionally equivalent to the proteins/genes of the subject invention can be used to transform hosts, including plants. Additional guidance regarding the production of synthetic genes can be found in, for example, U.S. Pat. No. 5,380,831.

US 11,371,055 B2

35

## 2.2—AAD-12 Plant Rebuild Analysis.

Extensive analysis of the 876 base pairs (bp) of the DNA sequence of the native AAD-12 coding region (SEQ ID NO: 1) revealed the presence of several sequence motifs that are thought to be detrimental to optimal plant expression, as well as a non-optimal codon composition. The protein encoded by SEQ ID NO:1 (AAD-12) is presented as SEQ ID NO:2. To improve production of the recombinant protein in monocots as well as dicots, a “plant-optimized” DNA sequence AAD-12 (v1) (SEQ ID NO:3) was developed that encodes a protein (SEQ ID NO:4) which is the same as the native SEQ ID NO:2 except for the addition of an alanine residue at the second position (underlined in SEQ ID NO:4). The additional alanine codon (GCT; underlined in SEQ ID NO:3) encodes part of an NcoI restriction enzyme recognition site (CCATGG) spanning the ATG translational start codon. Thus, it serves the dual purpose of facilitating subsequent cloning operations while improving the sequence context surrounding the ATG start codon to optimize translation initiation. The proteins encoded by the native and plant-optimized (v1) coding regions are 99.3% identical, differing only at amino acid number 2. In contrast, the native and plant-optimized (v1) DNA sequences of the coding regions are only 79.7% identical. Table 5 shows the differences in codon compositions of the native (Columns A and D) and plant-optimized sequences (Columns B and E), and allows comparison to a theoretical plant-optimized sequence (Columns C and F).

36

It is clear from examination of Table 5 that the native and plant-optimized coding regions, while encoding nearly identical proteins, are substantially different from one another. The Plant-Optimized version (v1) closely mimics the codon composition of a theoretical plant-optimized coding region encoding the AAD-12 protein.

2.3 Rebuild for *E. coli* Expression

Specially engineered strains of *Escherichia coli* and associated vector systems are often used to produce relatively large amounts of proteins for biochemical and analytical studies. It is sometimes found that a native gene encoding the desired protein is not well suited for high level expression in *E. coli*, even though the source organism for the gene may be another bacterial genus. In such cases it is possible and desirable to reengineer the protein coding region of the gene to render it more suitable for expression in *E. coli*. *E. coli* Class II genes are defined as those that are highly and continuously expressed during the exponential growth phase of *E. coli* cells. (Henaut, A. and Danchin, A. (1996) in *Escherichia coli and Salmonella typhimurium cellular and molecular biology*, vol. 2, pp. 2047-2066. Neidhardt, F., Curtiss III, R., Ingraham, J., Lin. E., Low, B., Magasanik, B., Reznikoff, W., Riley, M., Schaechter, M. and Umberger, H. (eds.) American Society for Microbiology, Washington, D.C.). Through examination of the codon compositions of the coding regions of *E. coli* Class II genes, one can devise an average codon composition for these *E. coli*-Class II gene coding regions. It is thought that a protein coding region

TABLE 5

Codon composition comparisons of coding regions of Native AAD-12, Plant-Optimized version (v1) and a Theoretical Plant-Optimized version.

Amino Acid	Codon	A	B	C	Amino Acid	Codon	D	E	F
		Native #	Plant Opt v1 #	Theor. Plant Opt. #			Native #	Plant Opt v1 #	Theor. Plant Opt. #
ALM (A)	GCA	1	10	11	LEU (L)	CTA	0	0	0
	GCC	35	16	15		CTC	1	8	8
	GCG	7	0	0		CTG	23	0	0
	GCT	0	18	17		CTT	0	8	8
ARG (R)	AGA	0	4	5		TTA	0	0	0
	AGG	0	4	6		TTG	0	8	8
	CGA	0	0	0	LYS (K)	AAA	1	1	2
	CGC	15	6	4		AAG	5	5	4
	CGG	3	0	0	MET (M)	ATG	10	10	10
	CGT	0	4	3		TTC	7	5	5
ASN (N)	AAC	3	2	2	PHE (F)	TTT	1	3	3
	AAT	1	2	2		CCA	0	5	6
ASP (D)	GAC	15	9	9		CCC	9	4	4
	GAT	2	8	8		CCG	5	0	0
CYS (C)	TGC	3	2	2		CCT	0	5	5
	TGT	0	1	1	SER (S)	AGC	5	4	3
END	TAA	1	0	1		AGT	0	0	0
	TAG	0	0			TCA	0	3	3
	TGA	0	1			TCC	2	3	3
GLN (Q)	CAA	1	8	7		TCG	6	0	0
	CAG	13	6	7		TCT	0	3	3
GLU (E)	GAA	3	4	4	THR (T)	ACA	1	4	5
	GAG	8	7	7		ACC	11	7	7
GLY (G)	GGA	0	8	7		ACG	5	0	0
	GGC	24	7	7		ACT	1	7	6
	GGG	1	3	4		TGG	8	8	8
	GGT	0	7	7		TAC	4	3	3
HIS (H)	CAC	8	9	9		TAT	1	2	2
	CAT	8	7	7	VAL (V)	GTA	0	0	0
ILE (I)	ATA	0	2	2		GTC	6	8	7
	ATC	10	4	5		GTG	18	8	9
	ATT	1	5	4		GTT	0	8	8
Totals		163	164	163	Totals		130	130	130

US 11,371,055 B2

37

having an average codon composition mimicking that of the Class II genes will be favored for expression during the exponential growth phase of *E. coli*. Using these guidelines, a new DNA sequence that encodes the AAD-12 protein (SEQ ID NO:4); including the additional alanine at the second position, as mentioned above), was designed according to the average codon composition of *E. coli* Class II gene coding regions. The initial sequence, whose design was based only on codon composition, was further engineered to include certain restriction enzyme recognition sequences suitable for cloning into *E. coli* expression vectors. Detrimental sequence features such as highly stable stemloop structures were avoided, as were intragenic sequences homologous to the 3' end of the 16S ribosomal RNA (i.e. Shine Dalgarno sequences) The *E. coli*-optimized sequence (v2) is disclosed as SEQ ID NO:5 and encodes the protein disclosed in SEQ ID NO:4.

The native and *E. coli*-optimized (v2) DNA sequences are 84.0% identical, while the plant-optimized (v1) and *E. coli*-optimized (v2) DNA sequences are 76.0% identical. Table 6 presents the codon compositions of the native AAD-12 coding region (Columns A and D), an AAD-12 coding region optimized for expression in *E. coli* (v2; Columns B and E) and the codon composition of a theoretical coding region for the AAD-12 protein having an optimal codon composition of *E. coli* Class II genes (Columns C and F).

38

identical proteins, are substantially different from one another. The *E. coli*-Optimized version (v2) closely mimics the codon composition of a theoretical *E. coli*-optimized coding region encoding the AAD-12 protein.

2.4—Design of a Soybean-Codon-Biased DNA Sequence Encoding a Soybean EPSPS Having Mutations that Confer Glyphosate Tolerance. This example teaches the design of a new DNA sequence that encodes a mutated soybean 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS), but is optimized for expression in soybean cells. The amino acid sequence of a triply-mutated soybean EPSPS is disclosed as SEQ ID NO:5 of WO 2004/009761. The mutated amino acids in the so-disclosed sequence are at residue 183 (threonine of native protein replaced with isoleucine), residue 186 (arginine in native protein replaced with lysine), and residue 187 (proline in native protein replaced with serine). Thus, one can deduce the amino acid sequence of the native soybean EPSPS protein by replacing the substituted amino acids of SEQ ID NO:5 of WO 2004/009761 with the native amino acids at the appropriate positions. Such native protein sequence is disclosed as SEQ ID NO:20 of PCT/US2005/014737 (filed May 2, 2005). A doubly mutated soybean EPSPS protein sequence, containing a mutation at residue 183 (threonine of native protein replaced with isoleucine), and at residue 187 (proline in native protein replaced with serine) is disclosed as SEQ ID NO:21 of PCT/US2005/014737.

TABLE 6

Codon composition comparisons of coding regions of Native AAD-12, <i>E. coli</i> -Optimized version(v2) and a Theoretical <i>E. coli</i> Class II-Optimized version.										
Amino Acid	Codon	A Native #	B <i>E. Coli</i> Opt v2 #	C Theor. Class II #	Amino Acid	Codon	D Native #	E <i>E. coli</i> Opt v2 #	F Theor. Class II #	
ALA (A)	GCA	1	13	13	LEU (L)	CTA	0	0	0	
	GCC	35	0	0		CTC	1	2	0	
	GCG	7	18	17		CTG	23	20	24	
	GCT	0	13	14		CTT	0	1	0	
ARG (R)	AGA	0	0	0	LYS (K)	TTA	0	1	0	
	AGG	0	0	0		TTG	0	0	0	
	CGA	0	0	0		AAA	1	4	5	
	CGC	15	6	6		AAG	5	2	1	
ASN (N)	CGG	3	0	0	MET (M)	ATG	10	10	10	
	CGT	0	12	12		PHE (E)	TTT	7	6	6
	AAC	3	4	4			TTT	1	2	2
	AAT	1	0	0			CCA	0	3	2
ASP (D)	GAC	15	10	9	PRO (P)		CCC	9	0	0
	GAT	2	7	8		CCG	5	11	12	
CYS (C)	TGC	3	2	2	SER (S)	CCT	0	0	0	
	TGT	0	1	1		AGC	5	4	4	
END	TAA	1	1	1		AGT	0	0	0	
	TAG	0	0	0		TCA	0	0	0	
	TGA	0	0	0		TCC	2	5	4	
GLN (Q)	CAA	1	3	3		TCG	6	0	0	
	CAG	13	11	11		TCT	0	4	5	
GLU (E)	GAA	3	8	8	THR (T)	ACA	1	0	0	
	GAG	8	3	3		ACC	11	12	12	
GLY (G)	GGA	0	0	0		ACG	5	0	0	
	GGC	24	12	11		ACT	1	6	6	
	GGG	1	0	0		TRP (W)	TGG	8	8	8
	GGT	0	13	14		TYR (Y)	TAC	4	3	3
HIS (H)	CAC	8	11	11	VAL (V)	TAT	1	2	2	
	CAT	8	5	5		GTA	0	6	6	
ILE (I)	ATA	0	0	0		GTC	6	0	0	
	ATC	10	7	7		GTG	18	8	7	
	ATT	1	4	4		GTT	0	10	11	
Totals		163	164	164	Totals		130	130	130	

It is clear from examination of Table 6 that the native and *E. coli*-optimized coding regions, while encoding nearly

A codon usage table for soybean (*Glycine max*) protein coding sequences, calculated from 362,096 codons (ap-

## US 11,371,055 B2

39

proximately 870 coding sequences), was obtained from the “kazusa.or.jp/codon” World Wide Web site. Those data were reformatted as displayed in Table 7. Columns D and H of Table 7 present the distributions (in % of usage for all codons for that amino acid) of synonymous codons for each amino acid, as found in the protein coding regions of soybean genes. It is evident that some synonymous codons for some amino acids (an amino acid may be specified by 1, 2, 3, 4, or 6 codons) are present relatively rarely in soybean protein coding regions (for example, compare usage of GCG and GCT codons to specify alanine). A biased soybean codon usage table was calculated from the data in Table 7. Codons found in soybean genes less than about 10% of total occurrences for the particular amino acid were ignored. To balance the distribution of the remaining codon choices for an amino acid, a weighted average representation for each codon was calculated, using the formula:

$$\text{Weighted \% of C1} = \frac{1}{(\% \text{ C1} + \% \text{ C2} + \% \text{ C3} + \text{etc.})} \times \% \text{ C1} \times 100$$

where C1 is the codon in question, C2, C3, etc. represent the remaining synonymous codons, and the % values for the relevant codons are taken from columns D and H of Table 7 (ignoring the rare codon values in bold font). The Weighted % value for each codon is given in Columns C and G of Table 7. TGA was arbitrarily chosen as the translation terminator. The biased codon usage frequencies were then entered into a specialized genetic code table for use by the OptGene™ gene design program (Ocimum Biosolutions LLC, Indianapolis, Ind.).

40

To derive a soybean-optimized DNA sequence encoding the doubly mutated EPSPS protein, the protein sequence of SEQ ID NO:21 from PCT/US2005/014737 was reverse-translated by the OptGene™ program using the soybean-biased genetic code derived above. The initial DNA sequence thus derived was then modified by compensating codon changes (while retaining overall weighted average representation for the codons) to reduce the numbers of CG and TA doublets between adjacent codons, increase the numbers of CT and TG doublets between adjacent codons, remove highly stable intrastrand secondary structures, remove or add restriction enzyme recognition sites, and to remove other sequences that might be detrimental to expression or cloning manipulations of the engineered gene. Further refinements of the sequence were made to eliminate potential plant intron splice sites, long runs of A/T or C/G residues, and other motifs that might interfere with RNA stability, transcription, or translation of the coding region in plant cells. Other changes were made to eliminate long internal Open Reading Frames (frames other than +1). These changes were all made within the constraints of retaining the soybean-biased codon composition as described above, and while preserving the amino acid sequence disclosed as SEQ ID NO:21 of PCT/US2005/014737.

The soybean-biased DNA sequence that encodes the EPSPS protein of SEQ ID NO:21 is disclosed as bases 1-1575 of SEQ ID NO:22 of PCT/US2005/014737. Synthesis of a DNA fragment comprising SEQ ID NO:22 of

TABLE 7

Synonymous codon representation in soybean protein coding sequences, and calculation of a biased codon representation set for soybean-optimized synthetic gene design.							
A Amino Acid	B Codon	C Weighted %	D Soybean %	E Amino Acid	F Codon	G Weighted %	H Soybean %
ALA (A)	GCA	33.1	30.3	LEU (L)	CTA	DNU	9.1
	GCC	24.5	22.5		CTC	22.4	18.1
	GCG	DNU *	8.5		CTG	16.3	13.2
	GCT	42.3	38.7		CTT	31.5	25.5
ARC (R)	AGA	36.0	30.9	LYS (K)	TTA	DNU	9.8
	AGG	32.2	27.6		TTG	29.9	24.2
	CGA	DNU	8.2		AAA	42.5	42.5
	CGC	14.8	12.7		AAG	57.5	57.5
ASN (N)	CGG	DNU	6.0	MET (M)	ATG	100.0	100
	CGT	16.9	14.5		TTC	49.2	49.2
	AAC	50.0	50.0		TTT	50.8	50.8
	AAT	50.0	50.0		CCA	39.8	36.5
ASP (D)	GAC	38.1	38.1	PRO (P)	CCC	20.9	19.2
	GAT	61.9	61.9		CCG	DNU	8.3
CYS (C)	TGC	50.0	50.0	SER (S)	CCT	39.3	36.0
	TGT	50.0	50.0		AGC	16.0	15.1
END	TAA	DNU	40.7	THR (T)	AGT	18.2	17.1
	TAG	DNU	22.7		TCA	21.9	20.6
	TGA	100.0	36.6		TCC	18.0	16.9
GLN (Q)	CAA	55.5	55.5	ICC	TCG	DNU	6.1
	CAG	44.5	44.5		TCT	25.8	24.2
GLU (E)	GAA	50.5	50.5	TRP (W)	ACA	32.4	29.7
	GAG	49.5	49.5		ACC	30.2	27.7
GLY (G)	GGA	31.9	31.9	TVR (Y)	ACG	DNU	8.3
	GGC	19.3	19.3		ACT	37.4	34.3
	GGG	18.4	18.4		TGG	100.0	100
	GGT	30.4	30.4		TAC	48.2	48.2
HIS (H)	CAC	44.8	44.8	VAL (V)	TAT	51.8	51.8
	CAT	55.2	55.2		GTA	11.5	11.5
ILE (I)	ATA	23.4	23.4		GTC	17.8	17.8
	ATC	29.9	29.9		GTG	32.0	32.0
	ATT	46.7	46.7		GTT	38.7	38.7

\*DNU = Do Not Use



US 11,371,055 B2

41

PCT/US2005/014737 was performed by a commercial supplier (PicoScript, Houston Tex.).

### Example 3—Cloning of Expression and Transformation Vectors

#### 3.1 Construction of *E. coli*, pET Expression Vector.

Using the restriction enzymes corresponding to the sites added with the additional cloning linkers (Xba 1, Xho 1) AAD-12 (v2) was cut out of the picoscript vector, and ligated into a pET280 streptomycin/spectinomycin resistant vector. Ligated products were then transformed into TOP10F<sup>+</sup> *E. coli*, and plated on to Luria Broth+50 µg/ml Streptomycin & Spectinomycin (LB S/S) agar plates.

To differentiate between AAD-12 (v2): pET280 and pCR2.1: pET280 ligations, approximately 20 isolated colonies were picked into 6 ml of LB-S/S, and grown at 37° C. for 4 hours with agitation. Each culture was then spotted onto LB+Kanamycin 50 µg/ml plates, which were incubated at 37° C. overnight. Colonies that grew on the LB-K were assumed to have the pCR2.1 vector ligated in, and were discarded. Plasmids were isolated from the remaining cultures as before, and checked for correctness with digestion by XbaI/XhoI. The final expression construct was given the designation pDAB3222.

#### 3.2—Construction of *Pseudomonas* Expression Vector

The AAD-12 (v2) open reading frame was initially cloned into the modified pET expression vector (Novagen), “pET280 S/S”, as an XbaI-XhoI fragment. The resulting plasmid pDAB725 was confirmed with restriction enzyme digestion and sequencing reactions. The AAD-12 (v2) open reading frame from pDAB725 was transferred into the *Pseudomonas* expression vector, pMYC1803, as an XbaI-XhoI fragment. Positive colonies were confirmed via restriction enzyme digestion. The completed construct pDAB739 was transformed into the MB217 and MB324 *Pseudomonas* expression strains.

42

#### 3.3—Completion of Binary Vectors.

The plant optimized gene AAD-12 (v1) was received from Picoscript (the gene rebuild design was completed (see above) and out-sourced to Picoscript for construction) and sequence verified (SEQ ID NO:3) internally, to confirm that no alterations of the expected sequence were present. The sequencing reactions were carried out with M13 Forward (SEQ ID NO:6) and M13 Reverse (SEQ ID NO:7) primers using the Beckman Coulter “Dye Terminator Cycle Sequencing with Quick Start Kit” reagents as before. Sequence data was analyzed and results indicated that no anomalies were present in the plant optimized AAD-12 (v1) DNA sequence. The AAD-12 (v1) gene was cloned into pDAB726 as an Nco I-Sac I fragment. The resulting construct was designated pDAB723, containing: [AtUbi10 promoter: Nt OSM 5'UTR: AAD-12 (v1): Nt OSM3'UTR: ORF1 polyA 3'UTR] (verified with a PvuII and a Not I restriction digests). A Not I-Not I fragment containing the described cassette was then cloned into the Not I site of the binary vector pDAB3038. The resulting binary vector, pDAB724, containing the following cassette [AtUbi10 promoter: Nt OSM5'UTR: AA4D-12 (v1): Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter: PAT: ORF25/26 3'UTR] was restriction digested (with Bam HI, Nco I, Not I, SacI, and Xmn I) for verification of the correct orientation. The verified completed construct (pDAB724) was used for transformation into *Agrobacterium* (see section 7.2).

#### 3.4—Cloning of Additional Transformation Constructs.

All other constructs created for transformation into appropriate plant species were built using similar procedures as previously described herein, and other standard molecular cloning methods (Maniatis et al., 1982). Table 8 lists all the transformation constructs used with appropriate promoters and features defined, as well as the crop transformed.

TABLE 8

Binary constructs used in transformation of various plant species.													
pDAB #	pDAS #	Species* Trans-formed	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Pro-moter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selec-tion gene	Promoter	Trxn Method
724	—	A, Ct, S	AAD12 v1	AtUbi10	NtOsm	—	—	—	Erythro-mycin	—	pat	CsVMV	Agro binary
3274	—	A	AAD12 v1	AtUbi10	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	—	—	Agro binary
3278	1580	T	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	pat	AtUbi10	Argo binary
3285	—	A	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	—	—	Spectino-mycin	—	pat	AtUbi10	Argo binary
3759	—	A, Ca, S	AAD12 v1	CsVMV	NtOsm	RB7 Mar v2	EPSPS	AtUbi10	Spectino-mycin	—	pat	AtUbi10	Argo binary
4101	1863	Cn, R	AAD12 v1	ZmUbi1	—	RB7 Mar v2	—	—	Ampicillin	—	AHAS v3	OsAct1	Whiskers/ Gun
4464	—	S	AAD12 v1	CsVMV	—	RB7 Mar v2	—	—	Spectino-mycin	—	pat	CsVMV	Argo binary
4468	—	S	AAD12 v1	AtUbi10	—	RB7 Mar v2	—	—	Spectino-mycin	—	pat	CsVMV	Argo binary
4472	—	S	AAD12 v1	AtUbi3	—	RB7 Mar v2	—	—	Spectino-mycin	—	pat	CsVMV	Argo binary
4476	—	S	AAD12 v1	ZmUbi1	—	RB7 Mar v2	—	—	Spectino-mycin	—	pat	CsVMV	Argo binary



US 11,371,055 B2

43

44

TABLE 8-continued

Binary constructs used in transformation of various plant species.													
pDAB #	pDAS #	Species* Trans-formed	Gene of interest (GOI)	Promoter	Feature 1	Feature 2	GOI 2	Pro-moter	Bacterial Selection gene	Bacterial Selection gene 2	Plant Selection gene	Promoter	Trxn Method
4480	—	S	AAD12 v1	AtAct2	—	RB7 Mar v2	—	—	Spectino-mycin	—	pat	CsVMV	Argo binary

\*A = *Arabidopsis*  
 ZmUbi1 = *Zea mays* Ubiquitin 1 Promoter  
 T = Tobacco  
 HptII = hygromycin phosphotransferase  
 S = Soybean  
 Ct = Cotton  
 R = Rice  
 Cn = Corn  
 Ca = Canola

CsVMV = Cassava Vein Mosaic Virus Promoter  
 AtUbi10 = *Arabidopsis thaliana* Ubiquitin 10 Promoter  
 Atubi3 = *Arabidopsis thaliana* Ubiquitin 3 Promoter  
 AtAct2 = *Arabidopsis thaliana* Actin 2 Promoter  
 RB7 Mar v2 = *Nicotiana tabacum* matrix associated region (MAR)  
 Nt Osm = *Nicotiana tabacum* Osmotin 5' Untranslated Region and the *Nicotiana tabacum* Osmotin 3' Untranslated Region

#### Example 4—Recombinant AAD-12 (v2) Expression and Purification in *Pseudomonas fluorescens*

##### 4.1—*Pseudomonas fluorescens* Fermentation

For shake flask experiment, 200 µl of *Pseudomonas fluorescens* strain MB324 glycerol stock carried AAD-12 (v2) construct pDAB739 (sec 3.2) was used to inoculate 50 ml fresh LB media supplemented with 30 µg/ml tetracycline/HCl. The culture (in a 250 ml baffled Erlenmeyer flask) was incubated on a shaker (New Brunswick Scientific Model Innova 44) at 300 rpm and 30° C. for 16 hrs. 20 ml of seed culture was transferred into 1 L *Pseudomonas fluorescens* culture media (Yeast extract, 5 g/L; K<sub>2</sub>HPO<sub>4</sub>, 5 g/L; (NH<sub>4</sub>)<sub>2</sub>PO<sub>4</sub>, 7.5 g/L; (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 1 g/L; MgSO<sub>4</sub>·7H<sub>2</sub>O, 1 g/L; KCl, 0.5 g/L; CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 g/L; NaCitrate·2H<sub>2</sub>O, 15 g/L; Glycerol, 95 g/L; Trace element solution, 10 mL/L; Trace element solution: FeCl<sub>3</sub>·6H<sub>2</sub>O, 5.4 g/L; MnCl<sub>2</sub>·4H<sub>2</sub>O, 1 g/L; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 1.45 g/L; CuSO<sub>4</sub>·5H<sub>2</sub>O, 0.25 g/L; H<sub>3</sub>BO<sub>3</sub>, 0.1 g/L; (NH<sub>4</sub>)<sub>2</sub>MO<sub>7</sub>O<sub>24</sub>, 0.1 g/L; concentrated HCl, 13 ml/L) supplemented with 20 µg/ml tetracycline/HCl and 250 µl of Pluronic L61(anti-foam) in a 2.8 L baffled Erlenmeyer flask. The cultures were incubated at 30° C. and 300 rpm for 24 hrs. Isopropyl β-D-1-thiogalacto-pyranoside (IPTG) was added to 1 mM final in the cultures and continued to incubate for approximately 48 hrs at 25° C. Cells were harvested by centrifugation at 7 krpm at 4° C. for 15 min, and cell paste was stored at -80° C. or immediately processed for purification.

For tank experiments, 1 ml each of the glycerol stock was inoculated a 1 L baffled flask containing 200 ml of LB media supplemented with 30 µg/ml tetracycline/HCl at 300 rpm and 32° C. for 16-24 hrs. The combined culture from three flasks (600 ml) was then aseptically transferred to a 20 L fermentor (B. Braun Bioreactor Systems) containing 10 L of Dow proprietary defined medium (through Teknova, Hollister, Calif.) designed to support high cell density growth. Growth temperature was maintained at 32° C. and the pH was controlled at the desired set-point through the addition of aqueous ammonia. Dissolved oxygen was maintained at a positive level in the liquid culture by regulating the sparged air flow and the agitation rates. The fed-batch fermentation process was carried out for approximately 24 hrs till cell density reached 170-200 OD<sub>575</sub>. IPTG was added to 1 mM to induce the recombinant protein expression and the temperature was reduced and maintained to 25° C. using circulation of cold-water supply. The induction phase of the

fermentation was allowed to continue for another 24 hrs. Samples (30 ml) were collected for various analyses to determine cell density and protein expression level at 6, 12, and 18 hrs post-induction time points. At the end of a fermentation run, cells were harvested by centrifugation at 10 krpm for 30 min. The cell pellets were frozen at -80° C. for further processing.

##### 4.2—Purification of AAD-12 (v2) for Biochemical Characterization and Antibody Production

Approximately 100-200 g of frozen (or fresh) *Pseudomonas* cells were thawed and resuspended in 1-2 L of extraction buffer containing 20 mM Tris-HCl, pH 8.5, and 25 ml of Protease inhibitor cocktail (Sigma cat #P8465). The cells were disrupted using Microfluidizer (model M110L or 110Y) (Microfluidics, Newton, Mass.) on ice with one pass at 11,000-12,000 psi. The lysate was centrifuged at 24,000 rpm for 20 min. The supernatant was transferred and dialyzed against 10 volumes of 20 mM Tris-HCl, pH 8.5 overnight at 4° C., or diafiltrated with this buffer and filtered through a 0.45 µm membrane before applying to the column separations. All subsequent protein separations were performed using Pharmacia AKTA Explorer 100 and operated at 4° C. Prior to loading, a Q Sepharose Fast Flow column (Pharmacia XK 50/00, 500 ml bed size) was equilibrated with 20 mM Tris-HCl, pH 8.5 buffer. The sample was applied to the column at 15 ml/min and then washed with this buffer until the eluate OD<sub>280</sub> returned to baseline. Proteins were eluted with 2 L of linear gradient from 0 to 0.3 M NaCl at a flow rate of 15 ml/min, while 45 ml fractions were collected. Fractions containing AAD-12 activity as determined by the colorimetric enzyme assay and also corresponding to the predicted molecular weight of AAD-12 protein (about 32 kDa band on SDS-PAGE) were pooled. Solid ammonium sulfate to final 0.5 M was added to the sample, and then applied to a Phenyl HP column (Pharmacia XK 50/20, 250 ml bed size) equilibrated in 0.5 M ammonium sulfate in 20 mM Tris-HCl, pH 8.0. This column was washed with the binding buffer at 10 ml/min until the OD<sub>280</sub> of the eluate returned to baseline, proteins were eluted within 2 column volumes at 10 ml/min by a linear gradient from 0.5 M to 0 M Ammonium sulfate in 20 mM Tris-HCl, pH 8.0, and 12.5 ml fractions were collected. The main peak fractions containing AAD-12 were pooled, and if necessary, concentrated using a MWCO 10 kDa cut-off membrane centrifugal filter device (Millipore). In some cases the sample was further applied to a Superdex 75 gel filtration

US 11,371,055 B2

45

column (Pharmacia XK 16/60, 110 ml bed size) with PBS buffer at a flow rate of 1 ml/min. Peak fractions containing pure AAD-12 were pooled and stored at  $-80^{\circ}\text{C}$ . In most cases, AAD-12 protein purity is approaching or above 99% after sequential ion-exchange column and hydrophobic interaction column two-step separation. A typical yield for purified AAD-12 is 12-18 mg/g of wet cells. Bulk protein sample was formulated in 20 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM DTT, and 1% Trehalose by diafiltration, and lyophilized on the Virtis Freezemobile Model 25EL (Virtis, Cardiner, N.Y.) for long-term storage.

Protein concentration was initially measured by Bradford assay using Bio-Rad Protein assay kit (cat #500-0006) with bovine serum albumin as standard. When needed, more accurate protein concentration was determined by using total amino acid hydrolysis. The sample was analyzed in Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, Calif.) with amino acid calibration standards (cat #PN5061-3330) purchased from Agilent.

AAD-12 activity was determined through out the processes to ensure no loss of the enzyme activity by each treatment and manipulation, as described in the Example 5 below. Protein purity was monitored by using SDS-PAGE and analytical size exclusion chromatography. Purified protein sample was further verified and confirmed by N-terminal amino acid sequencing, and shown consisting of expected AQTTLQITPT residues at its N-terminus. Short and long-term protein stability was tested by enzymatic activity and by native-PAGE and SDS-PAGE gel analysis under both non-reducing and reducing conditions. And it was noticed that AAD-12 is prone to oligomerization via disulfide bond formation, therefore typically 2 mM DTT was used for protein storage. Phosphate-buffer saline (PBS) and Tris-buffer saline (TBS) were tested for protein lyophilization, with and without the presence of 1% trehalose. Additionally, the endotoxin and DNA contaminant context from purified sample were measured respectively, and the integrity of the AAD-12 protein was also assessed by isoelectric focusing (IEF) analysis.

Ten milligrams of purified AAD-12 (v2) was delivered to Zymed Laboratories, Inc. (South San Francisco, Calif.) for rabbit polyclonal antibody production. The rabbit received 5 injections in the period of 5 weeks with each injection containing 0.5 mg of the purified protein suspended in 1 ml of complete Freund's Adjuvant. Sera were tested in both ELISA and Western blotting experiments to confirm specificity and affinity before affinity purification, and horseradish peroxidase (HRP) conjugation (Zymed Lab Inc).

#### Example 5—In Vitro Assays of AAD-12 Activity

##### 5.1—Assay Via Colorimetric Phenol Detection.

Enzyme activity was measured by colorimetric detection of the product phenol using a protocol modified from that of Fukumori and Hausinger (1993) (*J. Biol. Chem.* 268: 24311-24317) to enable deployment in a 96-well microplate format. The colorimetric assay has been described for use in measuring the activity of dioxigenases cleaving 2,4-D and dichlorprop to release the product 2,4-dichlorophenol. The color yield from several phenols was compared to that of 2,4-dichlorophenol using the detection method previously described to ascertain which phenol products could be readily detected. Phenols and phenol analogs were tested at a final concentration of 100  $\mu\text{M}$  in 0.15 ml 20 mM MOPS pH 6.75 containing 200  $\mu\text{M}$   $\text{NH}_4(\text{FeSO}_4)_2$ , 200  $\mu\text{M}$  sodium ascorbate. Pyridinols derived from fluroxypyr and triclopyr produced no significant color. The color yield of 2,4-dichlo-

46

rophanol was linear and proportional to the concentration of phenol in the assay up to  $\sim 500\text{ }\mu\text{M}$ . A calibration curve performed under standard assay conditions (160  $\mu\text{l}$  final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 17.2  $\mu\text{M}$  phenol.

Enzyme assays were performed in a total volume of 0.16 ml 20 mM MOPS pH 6.75 containing 200  $\mu\text{M}$   $\text{NH}_4\text{FeSO}_4$ , 200  $\mu\text{M}$  sodium ascorbate, 1 mM  $\alpha$ -ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or  $\alpha$ -ketoglutarate at time zero. After 5 minutes of incubation at  $25^{\circ}\text{C}$ , the reaction was terminated by addition of 30  $\mu\text{l}$  of a 1:1:1 mix of 50 mM Na EDTA; pH 10 buffer (3.09 g boric acid+3.73 g KCl+44 ml 1 N KOH) and 0.2% 4-aminoantipyrine. Then 10  $\mu\text{l}$  0.8% potassium ferricyanide was added and after 5 or 10 min, the absorbance at 510 nm was recorded in a spectrophotometric microplate reader. Blanks contained all reagents except for enzyme to account for the occasional slight contamination of some of the substrates by small amounts of phenols.

##### 5.2—Assay Via Detection of Chloropyridinol

AAD-12 action on potential substrates such as the herbicide triclopyr containing a substituted pyridine (rather than benzene rings) will release a pyridinol on cleavage of the aryloxyalkanoate bond. Pyridinols were not detected using the aminoantipyrine/ferricyanide phenol detection described in the preceding section. However, it was found that product chloropyridinols absorb strongly in the near UV with  $\lambda_{\text{max}}$  of 325 nm at pH 7 (extinction coefficient  $\sim 8,400\text{ M}^{-1}\text{cm}^{-1}$ ). This was used to create a continuous microplate-based spectrophotometric assay. Assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200  $\mu\text{M}$   $\text{NH}_4\text{FeSO}_4$ , 200  $\mu\text{M}$  sodium ascorbate, 1 mM  $\alpha$ -ketoglutarate, the appropriate substrate (added from a 100 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of the aryloxyalkanoate substrate, enzyme or  $\alpha$ -ketoglutarate at time zero and the increase in absorbance followed for 10 minutes at 325 nm in a microplate reader. The first 2 minutes of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200  $\mu\text{l}$  final assay volume) indicated that an absorbance at 510 nm of 0.1 was obtained from 11.9  $\mu\text{M}$  chloropyridinol.

##### 5.3—Colorimetric Assay Using 2-(2-Chloro,4-Nitrophenoxy)Propionate

A convenient assay of AAD-12 was devised using 2-(2-chloro,4-nitrophenoxy)propionate (CNPP) as substrate. Cleavage of CNPP by AAD-12 releases 2-chloro,4-nitrophenol. This phenol has a bright yellow absorbance at 410 nm at pH 7 enabling the reaction to be followed continuously or by endpoint analysis. The presence of AAD-12 activity can be monitored visually without the need for addition of further reagents. Microplate-based spectrophotometric assays were performed in a total volume of 0.2 ml 20 mM MOPS pH 6.75 containing 200  $\mu\text{M}$   $\text{NH}_4\text{FeSO}_4$ , 200  $\mu\text{M}$  sodium ascorbate, 1 mM  $\alpha$ -ketoglutarate, the appropriate amount of CNPP (added from a 10 mM stock made up in DMSO), and enzyme. Assays were initiated by addition of CNPP, enzyme, or  $\alpha$ -ketoglutarate at time zero and the increase in absorbance followed for 10 min at 410 nm in a microplate reader. The first 2 min of the reaction was used to determine initial rates. A calibration curve performed under standard assay conditions (200  $\mu\text{l}$  final assay volume) indicated that an absorbance at 410 nm of 0.1 was obtained from 25.1  $\mu\text{M}$  2-chloro, 4-nitrophenol. Using this assay, the

US 11,371,055 B2

47

kinetic constants for CNPP as a substrate were determined to be  $K_m=31\pm5.5\ \mu\text{M}$  and  $k=16.2\pm0.79\ \text{min}^{-1}$ .

#### Example 6—In Vitro Activity of AAD-12 on Various Substrates

6.1—AAD-12 (v2) Activity on (R,S)-Dichlorprop, (R)-Dichlorprop, (S)-Dichlorprop and 2,4-D

Using the phenol detection assay described in Example 5.1, four phenoxyalkanoates were assayed in a reaction mix containing 4.4  $\mu\text{g}$  purified AAD-12 (v2). (R,S)-dichlorprop (R,S-DP) was tested at 1 mM and (R)-dichlorprop, (S)-dichlorprop and 2,4-D were tested at 0.5 mM. The results are shown in FIG. 3, which illustrates activity of AAD-12 (v2) on 2,4-D and enantiomers of dichlorprop. 4.4  $\mu\text{g}$  AAD-12 (v2) was incubated with 0.5 mM substrate (1 mM for (R,S)-dichlorprop) and the reaction initiated by addition of  $\alpha$ -ketoglutarate. After 5 minutes, the reaction was quenched, and the absorbance at 510 nm determined after addition of colorimetric detection reagents. The background value without enzyme was subtracted.

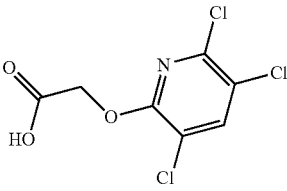
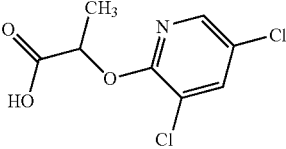
AAD-12 (v2) has excellent activity on (R,S)-dichlorprop and (S)-dichlorprop and has minimal activity on (R)-dichlorprop. This indicates that AAD-12 (v2) has a clear (S)-enantiomeric preference. The activity of AAD-12 (v2) on 2,4-D was equivalent to that on (S)-dichlorprop indicating that the enzyme can process oxypropionate and oxyacetates effectively.

6.2—AAD-12 (v2) Activity on Pyridyloxyalkanoates

Using the pyridinol assay described in Example 5.2, five pyridyloxyalkanoates were assayed at 1 mM in a reaction mix containing 6.8  $\mu\text{g}$  purified AAD-12 (v2). The rates of each reaction were monitored and are presented in Table 9. All five pyridyloxyalkanoates were cleaved to release pyridinols by AAD-12 (v2). The rates for the oxypropionate substrates 116844 and 91767 were somewhat faster than those for the corresponding acetates (triclopyr and 93833 respectively) indicating a preference of AAD-12 (v2) for oxypropionate over oxyacetate side chains. These data show that AAD-12 (v2) is able to effectively degrade pyridyloxyalkanoate herbicides such as triclopyr.

TABLE 9

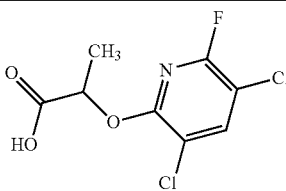
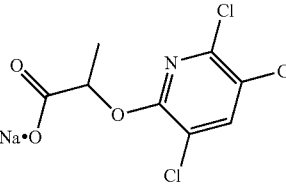
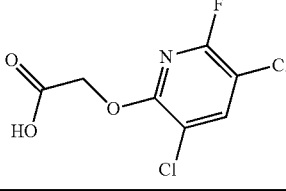
Rates of pyridyloxyalkanoate cleavage by AAD-12 (v2). 6.8  $\mu\text{g}$  AAD-12 (v2) was incubated with 1 mM substrate, the reaction initiated by addition of  $\alpha$ -ketoglutarate and the subsequent increase in absorbance monitored at 325 nm. The background rate of 1.4 mAU/min without  $\alpha$ -ketoglutarate was subtracted from the rates with substrate.

STRUCTURE	ID	Rate (mAU/min)	Rate relative to triclopyr
	triclopyr	97	1
	66357	225	2.3

48

TABLE 9-continued

Rates of pyridyloxyalkanoate cleavage by AAD-12 (v2). 6.8  $\mu\text{g}$  AAD-12 (v2) was incubated with 1 mM substrate, the reaction initiated by addition of  $\alpha$ -ketoglutarate and the subsequent increase in absorbance monitored at 325 nm. The background rate of 1.4 mAU/min without  $\alpha$ -ketoglutarate was subtracted from the rates with substrate.

STRUCTURE	ID	Rate (mAU/min)	Rate relative to triclopyr
	91767	190	0.8
	116844	257	1.4
	93833	118	0.5

6.3—Kinetic Constants of AAD-12 (v2) for 2,4-D, (R,S)-DCP and Triclopyr

The  $K_m$  and  $k_{cat}$  values of purified AAD-12 (v2) for the herbicides 2,4-D, (R,S)-dichlorprop and triclopyr were determined using the appropriate assay method. Substrate inhibition occurred at high concentrations ( $>1\ \text{mM}$ ) of 2,4-D and (R,S)-DCP so concentrations below this were used to fit the data to the Michaelis-Menten equation using Grafit 4.0 (Erihtacus Software, UK). No substrate inhibition was noted for triclopyr up to 2 mM. The kinetic constants are summarized in Table 10. From these data, the rate of AAD-12 (v2) cleavage of triclopyr is  $\sim 5\%$  that of 2,4-D, under maximal velocity conditions.

TABLE 10

Kinetic constants of AAD-12 (v2) for three herbicide substrates				
Substrate	$K_m\ \mu\text{M}$ ( $\pm\text{SE}$ )	$k_{cat}\ \text{min}^{-1}$ ( $\pm\text{SE}$ )	Assay method	Substrate inhibition at 2 mM
2,4-D	102 ( $\pm 18.4$ )	54.1 ( $\pm 3.1$ )	Phenol detection	55%
(R,S)-dichlorprop	122 ( $\pm 2.7$ )*	63.4 ( $\pm 0.5$ )	Phenol detection	55%
Triclopyr	241 ( $\pm 30$ )	2.6 ( $\pm 0.1$ )	$\Delta\text{A}325\ \text{nm}$	0%

\*Because of the (S)-enantiomeric preference of AAD-12 the  $K_m$  value was calculated assuming 50% of the racemic mixture was available as a substrate

#### Example 7—Transformation into *Arabidopsis* and Selection

7.1—*Arabidopsis thaliana* Growth Conditions.

Wildtype *Arabidopsis* seed was suspended in a 0.1% Agarose (Sigma Chemical Co., St. Louis, Mo.) solution. The

US 11,371,055 B2

49

suspended seed was stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination (stratification).

Sunshine Mix LP5 (Sun Gro Horticulture, Bellevue, Wash.) was covered with fine vermiculite and sub-irrigated with Hoagland's solution until wet. The soil mix was allowed to drain for 24 hours. Stratified seed was sown onto the vermiculite and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 7 days.

Seeds were germinated and plants were grown in a Conviron (models CMP4030 and CMP3244, Controlled Environments Limited, Winnipeg, Manitoba, Canada) under long day conditions (16 hours light/8 hours dark) at a light intensity of 120-150  $\mu\text{mol}/\text{m}^2 \text{ sec}$  under constant temperature (22° C.) and humidity (40-50%). Plants were initially watered with Hoagland's solution and subsequently with deionized water to keep the soil moist but not wet.

#### 7.2—*Agrobacterium* transformation.

An LB+agar plate with erythromycin (Sigma Chemical Co., St. Louis, Mo.) (200 mg/L) or spectinomycin (100 mg/L) containing a streaked DH5 $\alpha$  colony was used to provide a colony to inoculate 4 ml mini prep cultures (liquid LB+erythromycin). The cultures were incubated overnight at 37° C. with constant agitation. Qiagen (Valencia, Calif.) Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA.

Electro-competent *Agrobacterium tumefaciens* (strains Z707s, EHA101s, and LBA4404s) cells were prepared using a protocol from Weigel and Glazebrook (2002). The competent *Agrobacterium* cells were transformed using an electroporation method adapted from Weigel and Glazebrook (2002). 50  $\mu\text{l}$  of competent agro cells were thawed on ice and 10-25 ng of the desired plasmid was added to the cells. The DNA and cell mix was added to pre-chilled electroporation cuvettes (2 mm). An Eppendorf Electroporator 2510 was used for the transformation with the following conditions. Voltage: 2.4 kV, Pulse length: 5 msec.

After electroporation, 1 ml of YEP broth (per liter: 10 g yeast extract, 10 g Bacto-peptone, 5 g NaCl) was added to the cuvette, and the cell-YEP suspension was transferred to a 15 ml culture tube. The cells were incubated at 28° C. in a water bath with constant agitation for 4 hours. After incubation, the culture was plated on YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (Sigma Chemical Co., St. Louis, Mo.) (250 mg/L). The plates were incubated for 2-4 days at 28° C.

Colonies were selected and streaked onto fresh YEP+agar with erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) plates and incubated at 28° C. for 1-3 days. Colonies were selected for PCR analysis to verify the presence of the gene insert by using vector specific primers. Qiagen Spin Mini Preps, performed per manufacturer's instructions, were used to purify the plasmid DNA from selected *Agrobacterium* colonies with the following exception: 4 ml aliquots of a 15 ml overnight mini prep culture (liquid YEP+erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L)) were used for the DNA purification. An alternative to using Qiagen Spin Mini Prep DNA was lysing the transformed *Agrobacterium* cells, suspended in 10  $\mu\text{l}$  of water, at 100° C. for 5 minutes. Plasmid DNA from the binary vector used in the *Agrobacterium* transformation was included as a control. The PCR reaction was completed using Taq DNA polymerase from Takara Mirus Bio Inc. (Madison, Wis.) per manufacturer's instructions at 0.5 $\times$  concentrations. PCR reactions were carried out in a MJ Research Peltier Thermal Cycler programmed with the following conditions; 1) 94° C.

50

for 3 minutes, 2) 94° C. for 45 seconds, 3) 55° C. for 30 seconds, 4) 72° C. for 1 minute, for 29 cycles then 1 cycle of 72° C. for 10 minutes. The reaction was maintained at 4° C. after cycling. The amplification was analyzed by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. A colony was selected whose PCR product was identical to the plasmid control.

#### 7.3—*Arabidopsis* Transformation.

*Arabidopsis* was transformed using the floral dip method. The selected colony was used to inoculate one or more 15-30 ml pre-cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L). The culture(s) was incubated overnight at 28° C. with constant agitation at 220 rpm. Each pre-culture was used to inoculate two 500 ml cultures of YEP broth containing erythromycin (200 mg/L) or spectinomycin (100 mg/L) and streptomycin (250 mg/L) and the cultures were incubated overnight at 28° C. with constant agitation. The cells were then pelleted at approx. 8700 $\times g$  for 10 minutes at room temperature, and the resulting supernatant discarded. The cell pellet was gently resuspended in 500 ml infiltration media containing:  $\frac{1}{2}\times$  Murashige and Skoog salts/Gamberg's B5 vitamins, 10% (w/v) sucrose, 0.044  $\mu\text{M}$  benzylamino purine (10  $\mu\text{l}$ /liter of 1 mg/ml stock in DMSO) and 300  $\mu\text{l}$ /liter Silwet L-77. Plants approximately 1 month old were dipped into the media for 15 seconds, being sure to submerge the newest inflorescence. The plants were then laid down on their sides and covered (transparent or opaque) for 24 hours, then washed with water, and placed upright. The plants were grown at 22° C., with a 16-hour light/8-hour dark photoperiod. Approximately 4 weeks after dipping, the seeds were harvested.

#### 7.4—Selection of Transformed Plants.

Freshly harvested T<sub>1</sub> seed [AAD-12 (v1) gene] was allowed to dry for 7 days at room temperature. T<sub>1</sub> seed was sown in 26.5 $\times$ 51-cm germination trays (T.O. Plastics Inc., Clearwater, Minn.), each receiving a 200 mg aliquots of stratified T<sub>1</sub> seed (~10,000 seed) that had previously been suspended in 40 ml of 0.1% agarose solution and stored at 4° C. for 2 days to complete dormancy requirements and ensure synchronous seed germination.

Sunshine Mix LP5 (Sun Gro Horticulture Inc., Bellevue, Wash.) was covered with fine vermiculite and subirrigated with Hoagland's solution until wet, then allowed to gravity drain. Each 40 ml aliquot of stratified seed was sown evenly onto the vermiculite with a pipette and covered with humidity domes (KORD Products, Bramalea, Ontario, Canada) for 4-5 days. Domes were removed 1 day prior to initial transformant selection using glufosinate postemergence spray (selecting for the co-transformed PAT gene).

Seven days after planting (DAP) and again 11 DAP, T<sub>1</sub> plants (cotyledon and 2-4-leaf stage, respectively) were sprayed with a 0.2% solution of Liberty herbicide (200 g ai/L glufosinate, Bayer Crop Sciences, Kansas City, Mo.) at a spray volume of 10 ml/tray (703 L/ha) using a DeVilbiss compressed air spray tip to deliver an effective rate of 280 g ai/ha glufosinate per application. Survivors (plants actively growing) were identified 4-7 days after the final spraying and transplanted individually into 3-inch pots prepared with potting media (Metro Mix 360). Transplanted plants were covered with humidity domes for 3-4 days and placed in a 22° C. growth chamber as before or moved to directly to the greenhouse. Domes were subsequently removed and plants reared in the greenhouse (22 $\pm$ 5° C., 50 $\pm$ 30% RH, 14 h light:10 dark, minimum 500  $\mu\text{E}/\text{m}^2 \text{ s}^{-1}$  natural+supplemental light) at least 1 day prior to testing for



US 11,371,055 B2

## 51

the ability of AAD-12 (v1) (plant optimized gene) to provide phenoxy auxin herbicide resistance.

T<sub>1</sub> plants were then randomly assigned to various rates of 2,4-D. For *Arabidopsis*, 50 g ae/ha 2,4-D is an effective dose to distinguish sensitive plants from ones with meaningful levels of resistance. Elevated rates were also applied to determine relative levels of resistance (50, 200, 800, or 3200 g ac/ha). Tables 10 and 11 show comparisons drawn to an aryloxyalkanoate herbicide resistance gene (AAD-1 (v3)) previously described in PCT/US2005/014737.

All auxin herbicide applications were made using the DeVilbiss sprayer as described above to apply 703 L/ha spray volume (0.4 ml solution/3-inch pot) or applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was either technical grade (Sigma, St. Louis, Mo.) dissolved in DMSO and diluted in water (<1% DMSO final concentration) or the commercial dimethylamine salt formulation (456 g ac/L, NuFarm, St Joseph, Mo.). Dichlorprop used was commercial grade formulated as potassium salt of R-dichlorprop (600 g ai/L, AH Marks). As herbicide rates increased beyond 800 g ae/ha, the pH of the spray solution became exceedingly acidic, burning the leaves of young, tender *Arabidopsis* plants and complicating evaluation of the primary effects of the herbicides. It became standard practice to apply these high rates of herbicides in 200 mM HEPES buffer, pH 7.5.

Some T<sub>1</sub> individuals were subjected to alternative commercial herbicides instead of a phenoxy auxin. One point of interest was determining whether the pyridyloxyacetate auxin herbicides, triclopyr and fluroxypyr, could be effectively degraded in planta. Herbicides were applied to T<sub>1</sub> plants with use of a track sprayer in a 187 L/ha spray volume. T<sub>1</sub> plants that exhibited tolerance to 2,4-D DMA were further accessed in the T<sub>2</sub> generation.

#### 7.5—Results of Selection of Transformed Plants.

The first *Arabidopsis* transformations were conducted using AAD-12 (v1) (plant optimized gene). T<sub>1</sub> transformants were first selected from the background of untransformed seed using a glufosinate selection scheme. Over 300,000 T<sub>1</sub> seed were screened and 316 glufosinate resistant plants were identified (PAT gene), equating to a transformation/selection frequency of 0.10% which lies in the normal range of selection frequency of constructs where PAT+Liberty are used for selection. T<sub>1</sub> plants selected above were subsequently transplanted to individual pots and sprayed with various rates of commercial aryloxyalkanoate herbicides. Table 11 compares the response of AAD-12 (v1) and control genes to impart 2,4-D resistance to *Arabidopsis* T<sub>1</sub> transformants. Response is presented in terms of % visual injury 2 WAT. Data are presented as a histogram of individuals exhibiting little or no injury (<20%), moderate injury (20-40%), or severe injury (>40%). Since each T<sub>1</sub> is an independent transformation event, one can expect significant variation of individual T<sub>1</sub> responses within a given rate. An arithmetic mean and standard deviation is presented for each treatment. The range in individual response is also indicated in the last column for each rate and transformation. PAT/Cry1F-transformed *Arabidopsis* served as an auxin-sensitive transformed control. The AAD-12 (v1) gene imparted herbicide resistance to individual T<sub>1</sub> *Arabidopsis* plants. Within a given treatment, the level of plant response varied greatly and can be attributed to the fact each plant represents an independent transformation event. Of important note, at each 2,4-D rate tested, there were individuals that were unaffected while some were severely affected. An overall population injury average by rate is presented in Table 11 simply to demonstrate the significant difference between the

## 52

plants transformed with AAD-12 (v1) versus the wildtype or PAT/Cry1F-transformed controls. Injury levels tend to be greater and the frequency of uninjured plants was lower at elevated rates up to 3,200 g ae/ha (or 6× field rate). Also at these high rates, the spray solution becomes highly acidic unless buffered. *Arabidopsis* grown mostly in the growth chamber has a very thin cuticle and severe burning effects can complicate testing at these elevated rates. Nonetheless, many individuals have survived 3,200 g ac/ha 2,4-D with little or no injury.

TABLE 11

AAD-12 (v1) transformed T <sub>1</sub> <i>Arabidopsis</i> response to a range of 2,4-D rates applied postemergence, compared to an AAD-1 v3 (T <sub>4</sub> ) homozygous resistant population, or a Pat-Cry1F transformed, auxin-sensitive control.					
% Injury					Std
Averages	<20%	20-40%	>40%	Ave	Dev
AAD-12 (v1) gene T <sub>1</sub> transformants					
Untreated control-buffer	6	0	0	0	0
50 g ae/ha 2,4-D	6	0	2	16	24
200 g ae/ha 2,4-D	6	1	1	11	18
800 g ae/ha 2,4-D	5	2	1	15	20
3200 g ae/ha 2,4-D	8	0	0	6	6
PAT/Cry1F (transformed control)					
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	4	1	5	31	16
200 g ae/ha 2,4-D	0	0	10	70	2
800 g ae/ha 2,4-D	0	0	10	81	8
3200 g ae/ha 2,4-D	0	0	10	91	2
Homozygous AAD-1 (v3) gene T <sub>4</sub> plants					
Untreated control-buffer	10	0	0	0	0
50 g ae/ha 2,4-D	10	0	0	0	0
200 g ae/ha 2,4-D	10	0	0	0	0
800 g ae/ha 2,4-D	10	0	0	0	0
3200 g ae/ha 2,4-D	9	1	0	2	6

Table 12 shows a similarly conducted dose response of T<sub>1</sub> *Arabidopsis* to the phenoxypropionic acid, dichlorprop. The data shows that the herbicidally active (R-) isomer of dichlorprop does not serve as a suitable substrate for AAD-12 (v1). The fact that AAD-1 will metabolize R-dichlorprop well enough to impart commercially acceptable tolerance is one distinguishing characteristic that separates the two genes. (Table 12). AAD-1 and AAD-12 are considered R- and S-specific  $\alpha$ -ketoglutarate dioxygenases, respectively.

TABLE 12

T <sub>1</sub> <i>Arabidopsis</i> response to a range of R-dichlorprop rates applied postemergence.					
% Injury					
Averages	<20%	20-40%	>40%	Ave	Std Dev
AAD-12 v1 gene					
Untreated control	6	0	0	0	0
50 g ae/ha R-dichlorprop	0	0	8	63	7
200 g ae/ha R-dichlorprop	0	0	8	85	10
800 g ae/ha R-dichlorprop	0	0	8	96	4
3200 g ae/ha R-dichlorprop	0	0	8	98	2
PAT/Cry1F					
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	0	10	0	27	2
200 g ae/ha R-dichlorprop	0	0	10	69	3

US 11,371,055 B2

53

TABLE 12-continued

T <sub>1</sub> <i>Arabidopsis</i> response to a range of R-dichlorprop rates applied postemergence.					
Averages	% Injury			Ave	Std Dev
	<20%	20-40%	>40%		
800 g ae/ha R-dichlorprop	0	0	10	83	6
3200 g ae/ha R-dichlorprop	0	0	10	90	2
Homozygous AAD-12 (v3) gene T4 plants					
Untreated control	10	0	0	0	0
50 g ae/ha R-dichlorprop	10	0	0	0	0
200 g ae/ha R-dichlorprop	10	0	0	0	0
800 g ae/ha R-dichlorprop	10	0	0	0	0
3200 g ae/ha R-dichlorprop	10	0	0	0	0

## 7.6—AAD-12 (v1) as a Selectable Marker.

The ability to use AAD-12 (v1) as a selectable marker using 2,4-D as the selection agent was analyzed initially with *Arabidopsis* transformed as described above. Approximately 50 T<sub>4</sub> generation *Arabidopsis* seed (homozygous for AAD-12 (v1)) were spiked into approximately 5,000 wild-type (sensitive) seed. Several treatments were compared, each tray of plants receiving either one or two application timings of 2,4-D in one of the following treatment schemes: 7 DAP, 11 DAP, or 7 followed by 11 DAP. Since all individuals also contained the PAT gene in the same transformation vector, AAD-12 selected with 2,4-D could be directly compared to PAT selected with glufosinate.

Treatments were applied with a DeVilbiss spray tip as previously described. Plants were identified as Resistant or Sensitive 17 DAP. The optimum treatment was 75 g ae/ha 2,4-D applied 7 and 11 days after planting (DAP), was equally effective in selection frequency, and resulted in less herbicidal injury to the transformed individuals than the Liberty selection scheme. These results indicate AAD-12 (v1) can be effectively used as an alternative selectable marker for a population of transformed *Arabidopsis*.

## 7.7—Heritability.

A variety of T<sub>1</sub> events were self-pollinated to produce T<sub>2</sub> seed. These seed were progeny tested by applying 2,4-D (200 g ae/ha) to 100 random T<sub>2</sub> siblings. Each individual T<sub>2</sub> plant was transplanted to 7.5-cm square pots prior to spray application (track sprayer at 187 L/ha applications rate). Seventy-five percent of the T<sub>1</sub> families (T<sub>2</sub> plants) segregated in the anticipated 3 Resistant:1 Sensitive model for a dominantly inherited single locus with Mendelian inheritance as determined by Chi square analysis (P>0.05).

Seed were collected from 12 to 20 T<sub>2</sub> individuals (T<sub>3</sub> seed). Twenty-five T<sub>3</sub> siblings from each of eight randomly-selected T<sub>2</sub> families were progeny tested as previously described. Approximately one-third of the T<sub>2</sub> families anticipated to be homozygous (non-segregating populations) have been identified in each line. These data show AAD-12 (v1) is stably integrated and inherited in a Mendelian fashion to at least three generations.

7.8—Additional Foliar Applications Herbicide Resistance in AAD-12 *Arabidopsis*.

The ability of AAD-12 (v1) to provide resistance to other aryloxyalkanoate auxin herbicides in transgenic *Arabidopsis* was determined by foliar application of various substrates. T<sub>2</sub> generation *Arabidopsis* seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 6.4). A transformed-control line containing PAT and the insect resistance gene Cry1F was planted in a similar manner. Seedlings were transferred to individual 3-inch pots in the

54

greenhouse. All plants were sprayed with the use of a track sprayer set at 187 L/ha. The plants were sprayed with a range of pyridyloxyacetate herbicides: 280-2240 g ae/ha triclopyr (Garlon 3A, Dow AgroSciences) and 280-2240 g ae/ha fluroxypyr (Starane, Dow AgroSciences); and the 2,4-D metabolite resulting from AAD-12 activity, 2,4-dichlorophenol (DCP, Sigma) (at a molar equivalent to 280-2240 g ac/ha of 2,4-D, technical grade DCP was used). All applications were formulated in water. Each treatment was replicated 3-4 times. Plants were evaluated at 3 and 14 days after treatment.

There is no effect of the 2,4-D metabolite, 2,4-dichlorophenol (DCP), on transgenic non-AAD-12 control *Arabidopsis* (Pat/Cry1F). AAD-12-transformed plants were also clearly protected from the triclopyr and fluroxypyr herbicide injury that was seen in the transformed non-resistant controls (see Table 13). These results confirm that AAD-12 (v) in *Arabidopsis* provides resistance to the pyridyloxyacetic auxins tested. This is the first report of an enzyme with significant activity on pyridyloxyacetic acid herbicides. No other 2,4-D degrading enzyme has been reported with similar activity.

TABLE 13

Comparison of T<sub>2</sub> AAD-12 (v1) and transformed control *Arabidopsis* plant response to various foliar-applied auxinic herbicides.

Pyridyloxyacetic auxins		
Ave % Injury 14DAT		
Herbicide Treatment	Segregating T <sub>2</sub> AAD-12 (v1) plants (pDAB724.01.1.20)	Pat/Cry1F-Control
280 g ae/ha Triclopyr	0	52
560 g ae/ha Triclopyr	3	58
1120 g ae/ha Triclopyr	0	75*
2240 g ae/ha Triclopyr	3	75*
280 g ae/ha Fluroxypyr	0	75*
560 g ae/ha Fluroxypyr	2	75*
1120 g ae/ha Fluroxypyr	3	75*
2240 g ae/ha Fluroxypyr	5	75*
Inactive DCP metabolite		
280 g ae/ha 2,4-DCP	0	0
560 g ae/ha 2,4-DCP	0	0
1120 g ae/ha 2,4-DCP	0	0
2240 g ae/ha 2,4-DCP	0	0

\*Plants in this experiment were stunted and severely epinastic, but remained green and did not receive injury ratings >75%.

7.9—Molecular Analysis of AAD-12 (v1) *Arabidopsis*.

Invader Assay (methods of Third Wave Agbio Kit Procedures) for PAT gene copy number analysis was performed with total DNA obtained from Qiagen DNeasy kit on multiple AAD-12 (v1) homozygous lines to determine stable integration of the plant transformation unit containing PAT and AAD-12 (v1). Analysis assumed direct physical linkage of these genes as they were contained on the same plasmid.

Results showed that all 2,4-D resistant plants assayed, contained PAT (and thus by inference, AAD-12 (v1)). Copy number analysis showed total inserts ranged from 1 to 5 copies. This correlates, too, with the AAD-12 (v1) protein expression data indicating that the presence of the enzyme yields significantly high levels of resistance to all commercially available phenoxyacetic and pyridyloxyacetic acids.

7.10—*Arabidopsis* Transformed with Molecular Stack of AAD-12 (v1) and a Glyphosate Resistance Gene.

T<sub>1</sub> *Arabidopsis* seed was produced, as previously described, containing the pDAB3759 plasmid (AAD-12 (v1)+EPSPS) which encodes a putative glyphosate resistance trait. T<sub>1</sub> transformants were selected using AAD-12 (v1) as the selectable marker as described in example 7.6. T<sub>1</sub>



US 11,371,055 B2

55

plants (individually transformed events) were recovered from the first selection attempt and transferred to three-inch pots in the greenhouse as previously described. Three different control *Arabidopsis* lines were also tested: wildtype Columbia-0, AAD-12 (v1)+PAT T<sub>4</sub> homozygous lines (pDAB724-transformed), and PAT+Cry1F homozygous line (transformed control). The pDAB3759 and pDAB724 transformed plants were pre-selected at the seedling stage for 2,4-D tolerance. Four days after transplanting, plants were evenly divided for foliar treatment by track sprayer as previously described with 0, 26.25, 105, 420, or 1680 g ae/ha glyphosate (Glyphomax Plus, Dow AgroSciences) in water. All treatments were replicated 5 to 20 times. Plants were evaluated 7 and 14 days after treatment.

Initial resistance assessment indicated plants tolerant to 2,4-D were subsequently tolerant to glyphosate when compared to the response of the three control lines. These results indicate that resistance can be imparted to plants to two herbicides with differencing modes of action, including 2,4-D and glyphosate tolerance, allowing application of both herbicides postemergence. Additionally, AAD-12+2,4-D was used effectively as a selectable marker for a true resistance selection.

TABLE 14

T <sub>1</sub> <i>Arabidopsis</i> response to a range of glyphosate rates applied postemergence (14 DAT).					
AAD-12 v1 gene + EPSPS + HptII (pDAB3759) (Averages)	% Injury			% Injury Ave	Std Dev
	<20%	20-40%	>40%		
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	13	2	1	11	16
105 g ae/ha glyphosate	10	1	5	34	38
420 g ae/ha glyphosate	5	6	5	44	37
1680 g ae/ha glyphosate	0	0	16	85	9
PAT/Cry1F Averages	% Injury			% Injury Ave	Std Dev
	<20%	20-40%	>40%		
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	67	7
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0
Wildtype (Col-0) Averages	% Injury			% Injury Ave	Std Dev
	<20%	20-40%	>40%		
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	75	13
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0
pDAB724 T <sub>4</sub> (PAT + AAD-12) Averages	% Injury			% Injury Ave	Std Dev
	<20%	20-40%	>40%		
Untreated control	5	0	0	0	0
26.25 g ae/ha glyphosate	0	0	5	66	8
105 g ae/ha glyphosate	0	0	5	100	0
420 g ae/ha glyphosate	0	0	5	100	0
1680 g ae/ha glyphosate	0	0	5	100	0

7.11—AAD-12 *Arabidopsis* Genetically Stacked with AAD-1 to Give Wider Spectrum of Herbicide Tolerance.

AAD-12 (v1) (pDAB724) and AAD-1 (v3) (pDAB721) plants were reciprocally crossed and F<sub>1</sub> seed was collected. Eight F<sub>1</sub> seeds were planted and allowed to grow to produce seed. Tissue samples were taken from the eight F<sub>1</sub> plants and subjected to Western analysis to confirm the presence of

56

both genes. It was concluded that all 8 plants tested expressed both AAD-1 and AAD-12 proteins. The seed was bulked and allowed to dry for a week before planting.

One hundred F<sub>2</sub> seeds were sown and 280 g ai/ha glufosinate was applied. Ninety-six F<sub>2</sub> plants survived glufosinate selection fitting an expected segregation ratio for two independently assorting loci for glufosinate resistance (15 R:1S). Glufosinate resistant plants were then treated with 560 g ae/ha R-dichlorprop+560 g ae/ha triclopyr, applied to the plants under the same spray regimen as used for the other testing. Plants were graded at 3 and 14 DAT. Sixty-three of the 96 plants that survived glufosinate selection also survived the herbicide application. These data are consistent with an expected segregation pattern (9R:6S) of two independently assorting dominant traits where each gene gives resistance to only one of the auxinic herbicides (either R-dichlorprop or triclopyr). The results indicate that AAD-12 (pDAB724) can be successfully stacked with AAD-1 (pDAB721), thus increasing the spectrum herbicides that may be applied to the crop of interest [(2,4-D+R-dichlorprop) and (2,4-D+fluroxypyr+triclopyr), respectively]. This could be useful to bring 2,4-D tolerance to a very sensitive species through conventional stacking of two separate 2,4-D resistance genes. Additionally, if either gene were used as a selectable marker for a third and fourth gene of interest through independent transformation activities, then each gene pair could be brought together through conventional breeding activities and subsequently selected in the F<sub>1</sub> generation through paired sprays with herbicides that are exclusive between the AAD-1 and AAD-12 enzymes (as shown with R-dichlorprop and triclopyr for AAD-1 and AAD-12, respectively, above).

Other AAD stacks are also within the scope of the subject invention. The TfdA protein discussed elsewhere herein (Streber et al.), for example, can be used together with the subject AAD-12 genes to impart novel spectrums of herbicide resistance in transgenic plants of the subject invention.

#### Example 8—WHISKERS-Mediated Transformation of Corn Using Imazethapyr Selection

##### 8.1—Cloning of AAD-12 (v1).

The AAD-12 (v1) gene was cut out of the intermediate vector pDAB3283 as an NcoI/SacI fragment. This was ligated directionally into the similarly cut pDAB3403 vector containing the ZmUbi1 monocot promoter. The two fragments were ligated together using T4 DNA ligase and transformed into DH5 $\alpha$  cells. Minipreps were performed on the resulting colonies using Qiagen's QIA Spin mini prep kit, and the colonies were digested to check for orientation. This first intermediate construct (pDAB4100) contains the ZmUbi1:AAD-12 (v1) cassette. This construct was digested with NotI and PvuI to liberate the gene cassette and digest the unwanted backbone. This was ligated to NotI cut pDAB2212, which contains the AHAS selectable marker driven by the Rice Actin promoter OsAct1. The final construct was designated pDAB4101 or pDAS1863, and contains ZmUbi1/AAD-12 (v1)/ZmPer5::OsActa/AHAS/LZm-Lip.

##### 8.2—Callus/Suspension Initiation.

To obtain immature embryos for callus culture initiation, F<sub>1</sub> crosses between greenhouse-grown Hi-II parents A and B (Armstrong et al. 1991) were performed. When embryos were 1.0-1.2 mm in size (approximately 9-10 days post-pollination), ears were harvested and surface sterilized by scrubbing with Liqui-Nox® soap, immersed in 70% etha-

US 11,371,055 B2

57

nol for 2-3 minutes, then immersed in 20% commercial bleach (0.1% sodium hypochlorite) for 30 minutes.

Ears were rinsed in sterile, distilled water, and immature zygotic embryos were aseptically excised and cultured on 15Ag10 medium (N6 Medium (Chu et al., 1975), 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 25 mM L-proline, 10 mg/L AgNO<sub>3</sub>, 2.5 g/L Gelrite, pH 5.8) for 2-3 weeks with the scutellum facing away from the medium. Tissue showing the proper morphology (Welter et al., 1995) was selectively transferred at biweekly intervals onto fresh 15Ag10 medium for about 6 weeks, then transferred to 4 medium (N6 Medium, 1.0 mg/L 2,4-D, 20 g/L sucrose, 100 mg/L casein hydrolysate (enzymatic digest), 6 mM L-proline, 2.5 g/L Gelrite, pH 5.8) at bi-weekly intervals for approximately 2 months.

To initiate embryogenic suspension cultures, approximately 3 ml packed cell volume (PCV) of callus tissue originating from a single embryo was added to approximately 30 ml of H9CP+ liquid medium (MS basal salt mixture (Murashige and Skoog, 1962), modified MS Vitamins containing 10-fold less nicotinic acid and 5-fold higher thiamine-HCl, 2.0 mg/L 2,4-D, 2.0 mg/L  $\alpha$ -naphthaleneacetic acid (NAA), 30 g/L sucrose, 200 mg/L casein hydrolysate (acid digest), 100 mg/L myo-inositol, 6 mM L-proline, 5% v/v coconut water (added just before subculture), pH 6.0). Suspension cultures were maintained under dark conditions in 125 ml Erlenmeyer flasks in a temperature-controlled shaker set at 125 rpm at 28° C. Cell lines typically became established within 2 to 3 months after initiation. During establishment, suspensions were subcultured every 3.5 days by adding 3 ml PCV of cells and 7 ml of conditioned medium to 20 ml of fresh H9CP+ liquid medium using a wide-bore pipette. Once the tissue started doubling in growth, suspensions were scaled-up and maintained in 500 ml flasks whereby 12 ml PCV of cells and 28 ml conditioned medium was transferred into 80 ml H9CP+ medium. Once the suspensions were fully established, they were cryopreserved for future use.

#### 8.3—Cryopreservation and Thawing of Suspensions.

Two days post-subculture, 4 ml PCV of suspension cells and 4 ml of conditioned medium were added to 8 ml of cryoprotectant (dissolved in H9CP+ medium without coconut water, 1 M glycerol, 1 M DMSO, 2 M sucrose, filter sterilized) and allowed to shake at 125 rpm at 4° C. for 1 hour in a 125 ml flask. After 1 hour 4.5 ml was added to a chilled 5.0 ml Corning cryo vial. Once filled individual vials were held for 15 minutes at 4° C. in a controlled rate freezer, then allowed to freeze at a rate of -0.5° C./minute until reaching a final temperature of -40° C. After reaching the final temperature, vials were transferred to boxes within racks inside a Cryoplus 4 storage unit (Forma Scientific) filled with liquid nitrogen vapors.

For thawing, vials were removed from the storage unit and placed in a closed dry ice container, then plunged into a water bath held at 40-45° C. until "boiling" subsided. When thawed, contents were poured over a stack of ~8 sterile 70 mm Whatman filter papers (No. 4) in covered 100x25 mm Petri dishes. Liquid was allowed to absorb into the filters for several minutes, then the top filter containing the cells was transferred onto GN6 medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 2.5 g/L Gelrite, pH 5.8) for 1 week. After 1 week, only tissue with promising morphology was transferred off the filter paper directly onto fresh GN6 medium. This tissue was subcultured every 7-14 days until 1 to 3 grams was available for suspension initiation into approximately 30 ml H9CP+ medium in 125 ml Erlenmeyer flasks. Three milliliters PCV was subcultured into fresh

58

H9CP+ medium every 3.5 days until a total of 12 ml PCV was obtained, at which point subculture took place as described previously.

#### 8.4—Stable Transformation

Approximately 24 hours prior to transformation, 12 ml PCV of previously cryopreserved embryogenic maize suspension cells plus 28 ml of conditioned medium was subcultured into 80 ml of GN6 liquid medium (GN6 medium lacking Gelrite) in a 500 ml Erlenmeyer flask, and placed on a shaker at 125 rpm at 28° C. This was repeated 2 times using the same cell line such that a total of 36 ml PCV was distributed across 3 flasks. After 24 hours the GN6 liquid media was removed and replaced with 72 ml GN6 S/M osmotic medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 45.5 g/L sorbitol, 45.5 g/L mannitol, 100 mg/L myo-inositol, pH 6.0) per flask in order to plasmolyze the cells. The flasks were placed on a shaker shaken at 125 RPM in the dark for 30-35 minutes at 28° C., and during this time a 50 mg/ml suspension of silicon carbide whiskers was prepared by adding the appropriate volume 8.1 ml of GN6 S/M liquid medium to -405 mg of pre-autoclaved, sterile silicon carbide whiskers (Advanced Composite Materials, Inc.).

After incubation in GN6 S/M, the contents of each flask were pooled into a 250 ml centrifuge bottle. Once all cells settled to the bottom, all but ~14 ml of GN6 S/M liquid was drawn off and collected in a sterile 1-L flask for future use. The pre-wetted suspension of whiskers was vortexed for 60 seconds on maximum speed and 8.1 ml was then added to the bottle, to which 170  $\mu$ g DNA was added as a last step. The bottle was immediately placed in a modified Red Devil 5400 commercial paint mixer and agitated for 10 seconds. After agitation, the cocktail of cells, media, whiskers and DNA was added to the contents of the 1-L flask along with 125 ml fresh GN6 liquid medium to reduce the osmoticant. The cells were allowed to recover on a shaker at 125 RPM for 2 hours at 28° C. before being filtered onto Whatman #4 filter paper (5.5 cm) using a glass cell collector unit that was connected to a house vacuum line.

Approximately 2 ml of dispersed suspension was pipetted onto the surface of the filter as the vacuum was drawn. Filters were placed onto 60x20 mm plates of GN6 medium. Plates were cultured for 1 week at 28° C. in a dark box.

After 1 week, filter papers were transferred to 60x20 mm plates of GN6 (3P) medium (N6 Medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 3  $\mu$ M imazethapyr from Pursuit® DG, 2.5 g/L Gelrite, pH 5.8). Plates were placed in boxes and cultured for an additional week.

Two weeks post-transformation, the tissue was embedded by scraping all cells on the plate into 3.0 ml of melted GN6 agarose medium (N6 medium, 2.0 mg/L 2,4-D, 30 g/L sucrose, 100 mg/L myo-inositol, 7 g/L Sea Plaque agarose, pH 5.8, autoclaved for only 10 minutes at 121° C.) containing 3  $\mu$ M imazethapyr from Pursuit® DG. The tissue was broken up and the 3 ml of agarose and tissue were evenly poured onto the surface of a 100x15 mm plate of GN6 (3P). This was repeated for all remaining plates. Once embedded, plates were individually sealed with Nescofilm® or Parafilm M®, and then cultured until putative isolates appeared.

#### 8.4.1—Protocol for Isolate Recovery and Regeneration.

Putatively transformed events were isolated off the Pursuit®-containing embedded plates approximately 9 weeks post-transformation by transferring to fresh selection medium of the same concentration in 60x20 mm plates. If sustained growth was evident after approximately 2-3 weeks, the event was deemed to be resistant and was submitted for molecular analysis.

US 11,371,055 B2

59

Regeneration was initiated by transferring callus tissue to a cytokinin-based induction medium, 28 (3P), containing 3  $\mu$ M imazethapyr from Pursuit® DG, MS salts and vitamins, 30.0 g/L sucrose, 5 mg/L BAP, 0.25 mg/L 2,4-D, 2.5 g/L Gelrite; pH 5.7. Cells were allowed to grow in low light ( $13 \mu\text{Em}^{-2}\text{s}^{-1}$ ) for one week, then higher light ( $40 \mu\text{Em}^{-2}\text{s}^{-1}$ ) for another week, before being transferred to regeneration medium, 36 (3P), which was identical to 28 (3P) except that it lacked plant growth regulators. Small (3-5 cm) plantlets were removed and placed into 150x25-mm culture tubes containing selection-free SHGA medium (Schenk and Hildebrandt basal salts and vitamins, 1972; 1 g/L myo-inositol, 10 g/L sucrose, 2.0 g/L Gelrite, pH 5.8). Once plantlets developed a sufficient root and shoot system, they were transplanted to soil in the greenhouse.

From 4 experiments, full plantlets, comprised of a shoot and root, were formed in vitro on the embedded selection plates under dark conditions without undergoing a traditional callus phase. Leaf tissue from nine of these “early regenerators” were submitted for coding region PCR and Plant Transcription Unit (PTU) PCR for the AAD-12 gene and gene cassette, respectively. All had an intact AAD-12 coding region, while 3 did not have a full-length PTU (Table 15). These “early regenerators” were identified as 4101 events to differentiate them from the traditionally-derived events, which were identified as “1283” events. Plants from 19 additional events, obtained via standard selection and regeneration, were sent to the greenhouse, grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T<sub>1</sub> seed. Some of the events appear to be clones of one another due to similar banding patterns following Southern blot, so only 14 unique events were represented. T<sub>0</sub> plants from events were tolerant 70 g/ha imazethapyr. Invader analysis (AHAS gene) indicated insertion complexity ranging from 1 to >10 copies. Thirteen events contained the complete coding region for AAD-12; however, further analysis indicated the complete plant transformation unit had not been incorporated for nine events. None of the compromised 1863 events were advanced beyond the T<sub>1</sub> stage and further characterization utilized the 4101 events.

#### 8.5—Molecular Analysis: Maize Materials and Methods.

##### 8.5.1—Tissue Harvesting DNA Isolation and Quantification.

Fresh tissue is placed into tubes and lyophilized at 4° C. for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ $\mu$ l.

##### 8.5.2—Invader Assay Analysis.

The DNA samples are diluted to 20 ng/ $\mu$ l then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix is then prepared using the provided oligo mix and MgCl<sub>2</sub> (Third Wave Technologies). An aliquot of 7.5  $\mu$ l is placed in each well of the Invader assay plate followed by an aliquot of 7.5  $\mu$ l of controls, standards, and 20 ng/ $\mu$ l diluted unknown samples. Each well is overlaid with 15  $\mu$ l of mineral oil (Sigma). The plates are then incubated at 63° C. for 1 hour and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of

60

known copy standards developed and validated with Southern blot analysis is used to identify the estimated copy of the unknown events.

8.5.3—Polymerase Chain Reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-12 (v1) PTU are Forward—GAACAGTTAGACATGGTCTAAAGG (SEQ ID NO:8) and Reverse—GCTGCAACACTGATAATGCCAAGTGG (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 63° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes.

Primers for AAD-12 (v1) Coding Region PCR are Forward—ATGGCTCAGACCACTCTCCAAA (SEQ ID NO:10) and Reverse—AGCTGCATCCATGCCAGGGA (SEQ ID NO: 11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

##### 8.5.4—Southern Blot Analysis.

Southern blot analysis is performed with genomic DNA obtained from Qiagen DNeasy kit. A total of 2  $\mu$ g of genomic leaf DNA or 10  $\mu$ g of genomic callus DNA is subjected to an overnight digestion using BSM I and SWA I restriction enzymes to obtain PTU data.

After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85% agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20xSSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200x100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

##### 8.6—Postemergence Herbicide Tolerance in AAD-12 Transformed to Corn.

Four T<sub>0</sub> events were allowed to acclimate in the greenhouse and were grown until 2-4 new, normal looking leaves had emerged from the whorl (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were grown at 27° C. under 16 hour light:8 hour dark

## US 11,371,055 B2

## 61

conditions in the greenhouse. Plants were then treated with commercial formulations of either Pursuit® (imazethapyr) or 2,4-D Amine 4. Pursuit®@ was sprayed to demonstrate the function of the selectable marker gene present within the events tested. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. Plants were sprayed with either a lethal dose of imazethapyr (70 g ae/ha) or a rate of 2,4-D DMA salt capable of significant injury to untransformed corn lines (2240 g ac/ha). A lethal dose is defined as the rate that causes >95% injury to the Hi-II inbred. Hi-II is the genetic background of the transformants of the present invention.

Several individuals were safened from the herbicides to which the respective genes were to provide resistance. The

## 62

individual clone '001' from event "001" (a.k.a., 4101(0)-001-001), however, did incur minor injury but recovered by 14 DAT. Three of the four events were moved forward and individuals were crossed with 5XH751 and taken to the next generation. Each herbicide tolerant plant was positive for the presence of the AAD-12 coding region (PCR assay) or the presence of the AHAS gene (Invader assay) for 2,4-D and imazethapyr-tolerant plants, respectively. AAD-12 protein was detected in all 2,4-D tolerant T<sub>0</sub> plants events containing an intact coding region. The copy number of the transgene(s) (AHAS, and by inference AAD-12) varied significantly from 1 to 15 copies. Individual T<sub>0</sub> plants were grown to maturity and cross-pollinated with a proprietary inbred line in order to produce T<sub>1</sub> seed.

TABLE 15

Characterization of T <sub>0</sub> corn plants transformed with AAD-12.						
Event	Spray Treatment	% Injury (14 DAT)	AAD-12 ELISA (ppm TSP)	AAD12 PCR (Coding Region)	AAD12 PCR (PTU)	AHAS Copy # (Invader)
4101(0)003.001	2240 g ae/ha 2,4-D	0	146.9	+	+	1
4101(0)003.003	2240 g ae/ha 2,4-D	0	153.5	+	+	1
4101(0)005.001	2240 g ac/ha 2,4-D	0	539.7	+	+	9
4101(0)005.0012	0 g ae/ha 2,4-D	0	562.9	+	+	7
4101(0)001.001	70 g ae/ha imazethapyr	5	170.7	+	+	6
4101(0)002.001	0 g ae/ha imazethapyr	0	105.6	+	—	2
4101(0)002.002	70 g ae/ha imazethapyr	0	105.3	+	—	2
4101(0)003.002	70 g ae/ha imazethapyr	0	0	+	band small than expected	15

35

8.7—Verification of High 2,4-D Tolerance in T<sub>1</sub> Corn.

T<sub>1</sub> AAD-12 (v1) seed were planted into 3-inch pots containing Metro Mix media and at 2 leaf stage were sprayed with 70 g ae/ha imazethapyr to eliminate nulls. Surviving plants were transplanted to 1-gallon pots containing Metro Mix media and placed in the same growth conditions as before. At V3-V4 stage the plants were sprayed in the track sprayer set to 187 L/ha at either 560 or 2240 g ac/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to 5XH751×Hi II control plants. A grading scale of 0-10 (no injury to extreme auxin injury) was developed to distinguish brace root injury. Brace Root grades were taken on 14DAT to show 2,4-D tolerance. 2,4-D causes brace root malformation, and is a consistent indicator of auxinic herbicide injury in corn. Brace root data (as seen in the table below) demonstrates that 2 of the 3 events tested were robustly tolerant to 2240 g ae/ha 2,4-D DMA. Event "pDAB4101(0)001.001" was apparently unstable; however, the other two events were robustly tolerant to 2,4-D and 2,4-D+imazethapyr or 2,-4D+glyphosate (see Table 16).

TABLE 16

Brace Root injury of AAD-12 (v1) transformed T<sub>1</sub> plants and Untransformed control corn plants. A scale of 0-10, 10 being the highest, was used for grading the 2,4-D DMA injury. Results are a visual average of four replications per treatment.

Herbicide	Untransformed Control	AAD-12 (v1) pDAB4101(0)003.003	AAD-12 (v1) pDAB4101(0)001.001	AAD-12 (v1) pDAB4101(0)005.001
		Average Brace Root Injury (0-10 Scale)		
0 g ae/ha 2,4-D DMA	0	0	0	0



US 11,371,055 B2

63

TABLE 16-continued

Brace Root injury of AAD-12 (v1) transformed T<sub>1</sub> plants and Untransformed control corn plants. A scale of 0-10, 10 being the highest, was used for grading the 2,4-D DMA injury. Results are a visual average of four replications per treatment.

Herbicide	Untransformed Control	AAD-12 (v1) pDAB4101(0)003.003	AAD-12 (v1) pDAB4101(0)001.001	AAD-12 (v1) pDAB4101(0)005.001
Average Brace Root Injury (0-10 Scale)				
2240 g ae/ha 2,4-D DMA	9	1	8	0

## 8.8 AAD-12 (v1) Heritability in Corn.

A progeny test was also conducted on seven AAD-12 (v1) T<sub>1</sub> families that had been crossed with 5XH751. The seeds were planted in three-inch pots as described above. At the 3 leaf stage all plants were sprayed with 70 g ae/ha imazethapyr in the track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Four out of the six lines tested segregated as a single locus, dominant Mendelian trait (1R:1S) as determined by Chi square analysis. Surviving plants were subsequently sprayed with 2,4-D and all plants were deemed tolerant to 2,4-D (rates >560 g ae/ha). AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species when reciprocally crossed to a commercial hybrid.

## 8.9—Stacking of AAD-12 (v1) to Increase Herbicide Spectrum

AAD-12 (v1) (pDAB4101) and elite Roundup Ready inbred (BE1146RR) were reciprocally crossed and F<sub>1</sub> seed was collected. The seed from two F<sub>1</sub> lines were planted and treated with 70 g ae/ha imazethapyr at the V2 stage to eliminate nulls. To the surviving plants, reps were separated and either treated with 1120 g ae/ha 2,4-D DMA+70 g ae/ha imazethapyr (to confirm presence of AHAS gene) or 1120 g ae/ha 2,4-D DMA+1680 g ae/ha glyphosate (to confirm the presence of the Round Up Ready gene) in a track sprayer calibrated to 187 L/ha. Plants were graded 3 and 16 DAT. Spray data showed that AAD-12 (v1) can be conventionally stacked with a glyphosate tolerance gene (such as the Roundup CP4-EPSPS gene) or other herbicide tolerance genes to provide an increased spectrum of herbicides that may be applied safely to corn. Likewise imidazolinone+2, 4-D+glyphosate tolerance was observed in F<sub>1</sub> plants and showed no negative phenotype by the molecular or breeding stack combinations of these multiple transgenes.

TABLE 17

Data demonstrating increase herbicide tolerance spectrum resulting from an F <sub>1</sub> stack of AAD-12 (v1) and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF).				
Herbicide	Untransformed Control	2P782 (Roundup Ready Control)	AAD-12 (v1) pDAB4101(0)003.R003.AF	AAD-12 (v1) pDAB4101(0)005.R001.AF
Average % Injury 16DAT				
0 g ae/ha 2,4-D DMA	0	0	0	0
1120 g ae/ha 2,4-D DMA	21	19	0	0
1120 g ae/ha 2,4-D DMA + 70 g ae/ha imazethapyr	100	100	5	1

64

TABLE 17-continued

Data demonstrating increase herbicide tolerance spectrum resulting from an F <sub>1</sub> stack of AAD-12 (v1) and BE1146RR (an elite glyphosate tolerant inbred abbreviated as AF).				
Herbicide	Untransformed Control	2P782 (Roundup Ready Control)	AAD-12 (v1) pDAB4101(0)003.R003.AF	AAD-12 (v1) pDAB4101(0)005.R001.AF
Average % Injury 16DAT				
01120 g ae/ha 2,4-D DMA + 1680 g ae/ha glyphosate	100	71	2	5

## 8.10—Field Tolerance of pDAB4101 Transformed Corn Plants to 2,4-D, Triclopyr and Fluroxypyr Herbicides.

Field level tolerance trials were conducted on two AAD-12 (v1) pDAB4101 events (4101(0)003.R.003.AF and 4101(0)005.R001.AF) and one Roundup Ready (RR) control hybrid (2P782) at Fowler, Ind., and Wayside, Miss. Seeds were planted with cone planter on 40-inch row spacing at Wayside and 30 inch spacing at Fowler. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 1120, 2240 and 4480 g ae/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. The AAD-12 (v1) events contained the AHAS gene as a selectable marker. The F<sub>2</sub> corn events were segregating so the AAD-12 (v1) plants were treated with imazethapyr at 70 g ae/ha to remove the null plants. Herbicide treatments were applied when corn reached the V6 stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after treatment. Brace root injury ratings were taken at 28DAT on a scale of 0-10 with 0-1 being slight brace root fusing, 1-3 being moderate brace root swelling/wandering and root proliferation, 3-5 being moderate brace root fusing, 5-9 severe brace root fusing and malformation and 10 being total inhibition of brace roots.

AAD-12 (v1) event response to 2,4-D, triclopyr, and fluroxypyr at 14 days after treatment are shown in Table 18. Crop injury was most severe at 14 DAT. The RR control corn (2P782) was severely injured (44% at 14 DAT) by 2,4-D at 4480 g ae/ha, which is 8 times (8x) the normal field use rate. The AAD-12 (v1) events all demonstrated excellent tolerance to 2,4-D at 14 DAT with 0% injury at the 1, 2 and 4x rates, respectively. The control corn (2P782) was severely injured (31% at 14 DAT) by the 2x rate of triclopyr (840 g ae/ha). AAD-12 (v1) events demonstrated tolerance at 2x rates of triclopyr with an average of 3% injury at 14 DAT across the two events. Fluroxypyr at 280 g ae/ha caused 11% visual injury to the wild-type corn at 14 DAT. AAD-12 (v1) events demonstrated increased tolerance with an average of 8% injury at 5 DAT.

US 11,371,055 B2

65

Applications of auxinic herbicides to corn in the V6 growth stage can cause malformation of the brace roots. Table 18 shows the severity of the brace root injury caused by 2,4-D, triclopyr, and fluroxypyr. Triclopyr at 840 g ae/ha caused the most severe brace root fusing and malformation resulting in an average brace root injury score of 7 in the 2P782 control-type corn. Both AAD-12 (v1) corn events showed no brace root injury from the triclopyr treatment. Brace root injury in 2P782 corn increased with increasing rates of 2,4-D. At 4480 g ae/ha of 2,4-D, the AAD-12 events showed no brace root injury; whereas, severe brace root fusing and malformation was seen in the 2P782 hybrid. Fluroxypyr caused only moderate brace root swelling and wandering in the wild-type corn with the AAD-12 (v1) events showing no brace root injury.

This data clearly shows that AAD-12(v1) conveys high level tolerance in corn to 2,4-D, triclopyr and fluroxypyr at rates far exceeding those commercially used and that cause non-AAD-12 (v1) corn severe visual and brace root injury.

TABLE 18

Visual Injury of AAD-12 events and wild-type corn following foliar applications of 2,4-D, triclopyr and fluroxypyr under field conditions.				
Treatment	Rate (g ae/ha)	% Visual Injury 14DAT		
		AAD-12 4101(0)003.R.003.AF	AAD- 124101(0)005.001.AF	2P782 control
Untreated	0	0	0	0
2,4-D	1120	0	0	9
2,4-D	2240	0	1	20
2,4-D	4480	0	1	34
Fluroxypyr	280	1	5	11
Triclopyr	840	3	4	31
Dicamba	840	8	8	11

TABLE 19

Brace root injury ratings for AAD-12 and wild-type corn plants in response to 2,4-D, triclopyr and fluroxypyr under field conditions.				
Treatment	Rate (g ae/ha)	Brace Root Injury Rating (0-10 scale) 28DAT		
		AAD-12 event 4101(0)003.R.003.AF	AAD-12 event 4101(0)005.001.AF	Wild- type NK603
Untreated	0	0	0	0
2,4-D	1120	0	0	3
2,4-D	2240	0	0	5
2,4-D	4480	0	0	6
Fluroxypyr	280	0	0	2
Triclopyr	840	0	0	7
Dicamba	840	1	1	1

#### Example 9. Protein Detection from Transformed Plants Via Antibody

##### 9.1—Extracting AAD-12 (v1) from Plant Leaves.

Approximately 50 to 100 mg of leaf tissue was cut into small pieces (or 4 single-hole-punched leaf discs) and put into 2-ml cluster tubes containing 2 stainless steel BB beads (4.5 mm; Daisy Co., cat. #145462-000). Five hundred microliters of plant extraction buffer (PBS containing 0.05% Tween 20 and 1% Bovine serum albumin) was added to each sample. The tubes were capped and secured in the Geno/Grinder (Model 2000-115, Certiprep, Metuchen, N.J.) and shaken for 6 min with setting at 1x of 500 rpm. Tubes were

66

centrifuged at 5000xg for 10 min and supernatant containing the soluble proteins were analyzed for AAD-12 (v1) using Western Blots and ELISA.

##### 9.2—Enzyme Linked Immuno-Sorbent Assay (ELISA).

The assay was conducted at room temperature unless otherwise stated. One hundred micro-liter of purified anti-AAD-12 antibody (0.5 µg/ml) was coated on 96-well micro-titer well and incubated at 4° C. for 16 hours. The plate was washed four times with washing buffer (100 mM phosphate buffered saline (PBS; pH 7.4) containing 0.05% Tween 20) using a plate washer, followed by blocking with 4% skim milk dissolved in PBS for 1 hour. After washing, 100 µL standard AAD-12 of known concentrations or plant extracts from different samples were incubated in the wells. For standard curve, purified AAD-12 was diluted 2-fold serially from 52 to 0.813 ng/ml in triplicates. Plant extracts were diluted 5, 10, 20, and 40-fold in PBS and analyzed in duplicates. After 1 hour incubation, the plate was washed as above. One hundred micro-liter anti-AAD-12 antibody-HRP conjugate (0.5 ug/ml) was incubated in each well for 1 hour before washing. One hundred micro-liter HRP substrate, 1-Step™ Ultra TMB-ELISA (Pierce, Rockford, Ill.), was incubated in each well for 10 minutes before the reaction was stopped by adding 100 µL 0.4N H<sub>2</sub>SO<sub>4</sub>. The OD of each well was measured using a microplate reader at 450 nm. To determine the concentrations of AAD-12 (v1) in plant extract, the OD value of duplicates were averaged and extrapolated from the standard curve using the Softmax® Pro ver. 4.0 (Molecular Devices).

For comparison, each sample was normalized with its fresh weight and percent expression was calculated.

##### 9.3—Western Blotting Analysis.

Plant extracts or AAD-12 standards (5 and 0.5 µg/ml) were incubated with Laemmli sample buffer at 95° C. for 10 minutes and electrophoretically separated in 8-16% Tris-Glycine Precast gel. Proteins were then electro-transferred onto nitrocellulose membrane using standard protocol. After blocking in 4% skim milk in PBS, AAD-12 (v1) protein was detected by anti-AAD-12 antiserum followed by goat anti-rabbit/HRP conjugates. The detected protein was visualized by chemiluminescence substrate ECL Western Analysis Reagent (Amersham, N.J.).

#### Example 10—Tobacco Transformation

Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar, but not identical, to published methods (Horsch et al., 1988). To provide source tissue for the transformation, tobacco seed (*Nicotiana tabacum* cv. KY160) was surface sterilized and planted on the surface of TOB-medium, which is a hormone-free Murashige and Skoog medium (Murashige and Skoog, 1962) solidified with agar. Plants were grown for 6-8 weeks in a lighted incubator room at 28-30° C. and leaves collected sterily for use in the transformation protocol. Pieces of approximately one square centimeter were sterily cut from these leaves, excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB3278, aka pDAS1580, AAD-12 (v1)+PAT), grown overnight in a flask on a shaker set at 250 rpm at 28° C., were pelleted in a centrifuge and resuspended in sterile Murashige & Skoog salts, and adjusted to a final optical density of 0.5 at 600 nm. Leaf pieces were dipped in this bacterial suspension for approximately 30 seconds, then blotted dry on sterile paper towels and placed right side up on TOB+ medium (Murashige and Skoog medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark



US 11,371,055 B2

67

at 28° C. Two days later the leaf pieces were moved to TOB+ medium containing 250 mg/L cefotaxime (Agri-Bio, North Miami, Fla.) and 5 mg/L glufosinate ammonium (active ingredient in Basta, Bayer Crop Sciences) and incubated at 28-30° C. in the light. Leaf pieces were moved to fresh TOB+ medium with cefotaxime and Basta twice per week for the first two weeks and once per week thereafter. Four to six weeks after the leaf pieces were treated with the bacteria, small plants arising from transformed foci were removed from this tissue preparation and planted into medium TOB-containing 250 mg/L cefotaxime and 10 mg/L Basta in Phytatray™ II vessels (Sigma). These plantlets were grown in a lighted incubator room. After 3 weeks, stem cuttings were taken and re-rooted in the same media. Plants were ready to send out to the greenhouse after 2-3 additional weeks.

Plants were moved into the greenhouse by washing the agar from the roots, transplanting into soil in 13.75 cm square pots, placing the pot into a Ziploc® bag (SC Johnson & Son, Inc.), placing tap water into the bottom of the bag, and placing in indirect light in a 30° C. greenhouse for one week. After 3-7 days, the bag was opened; the plants were fertilized and allowed to grow in the open bag until the plants were greenhouse-acclimated, at which time the bag was removed. Plants were grown under ordinary warm greenhouse conditions (30° C., 16 hour day, 8 hour night, minimum natural+supplemental light=500  $\mu\text{E}/\text{m}^2 \text{ s}^{-1}$ ).

Prior to propagation, T<sub>0</sub> plants were sampled for DNA analysis to determine the insert copy number. The PAT gene which was molecularly linked to AAD-12 (v1) was assayed for convenience. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ $\mu\text{L}$ .

The DNA samples were diluted to 9 ng/ $\mu\text{L}$  and then denatured by incubation in a thermocycler at 95° C. for 10 minutes. Signal Probe mix was then prepared using the provided oligo mix and MgCl<sub>2</sub> (Third Wave Technologies). An aliquot of 7.5  $\mu\text{L}$  was placed in each well of the Invader assay plate followed by an aliquot of 7.5  $\mu\text{L}$  of controls, standards, and 20 ng/ $\mu\text{L}$  diluted unknown samples. Each well was overlaid with 15  $\mu\text{L}$  of mineral oil (Sigma). The plates were then incubated at 63° C. for 1.5 hours and read on the fluorometer (Biotek). Calculation of % signal over background for the target probe divided by the % signal over background internal control probe will calculate the ratio. The ratio of known copy standards developed and validated with southern blot analysis was used to identify the estimated copy of the unknown events.

All events were also assayed for the presence of the AAD-12 (v1) gene by PCR using the same extracted DNA samples. A total of 100 ng of total DNA was used as template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit. Primers for the Plant Transcription Unit (PTU) PCR AAD-12 were (SdpacodF: ATGGCTCA TGCTGCCCTCAGCC) (SEQ ID NO:12) and (SdpacodR: CGGGCAGGCCTAACTCCACC AA) (SEQ ID NO: 13). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 64° C. for 30 seconds, and 72° C. for 1

68

minute and 45 seconds followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. Four to 12 clonal lineages from each of 18 PCR positive events with 1-3 copies of PAT gene (and presumably AAD-12 (v1) since these genes are physically linked) were regenerated and moved to the greenhouse.

10.1 Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T<sub>0</sub> Tobacco

T<sub>0</sub> plants from each of the 19 events were challenged with a wide range of 2,4-D, triclopyr, or fluroxypyr sprayed on plants that were 3-4 inches tall. Spray applications were made as previously described using a track sprayer at a spray volume of 187 L/ha. 2,4-D dimethylamine salt (Riverside Corp) was applied at 0, 140, 560, or 2240 g ae/ha to representative clones from each event mixed in deionized water. Fluroxypyr was likewise applied at 35, 140, or 560 g ac/ha. Triclopyr was applied at 70, 280, or 1120 g ae/ha. Each treatment was replicated 1-3 times. Injury ratings were recorded 3 and 14 DAT. Every event tested was more tolerant to 2,4-D than the untransformed control line KY160. In several events, some initial auxinic herbicide-related epinasty occurred at doses of 560 g ae/ha 2,4-D or less. Some events were uninjured at 2,4-D applied at 2240 g ac/ha (equivalent to 4 $\times$  field rate). On the whole, AAD-12 (v1) events were more sensitive to fluroxypyr, followed by triclopyr, and least affected by 2,4-D. The quality of the events with respect to magnitude of resistance was discerned using T<sub>0</sub> plant responses to 560 g ae/ha fluroxypyr. Events were categorized into "low" (>40% injury 14 DAT), "medium" (20-40% injury), "high" (<20% injury). Some events were inconsistent in response among replicates and were deemed "variable."

TABLE 20

Tobacco T <sub>0</sub> events transformed with pDAS1580 (AAD-12 (v1) + PAT)						
# Tube	Plant ID	Copy # PAT	PTU PCR AAD-12	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance @
1	1580[1]-001	6	+			Not tested
2	1580[1]-002	8	+			Not tested
3	1580[1]-003	10	+			Not tested
4	1580[1]-004	1	+		*	High
5	1580[1]-005	2	+	*		Variable
6	1580[1]-006	6	+			Not tested
7	1580[1]-007	4	+			Not tested
8	1580[1]-008	3	+			Variable
9	1580[1]-009	4	+			Not tested
10	1580[1]-010	8	+			Not tested
11	1580[1]-011	3	+			High
12	1580[1]-012	12	+			Not tested
13	1580[1]-013	13	+			Not tested
14	1580[1]-014	4	+			Not tested
15	1580[1]-015	2	+	*		High
16	1580[1]-016	1 ?	+	*	*	High
17	1580[1]-017	3	+			High
18	1580[1]-018	1	+	*	*	Variable
19	1580[1]-019	1	+	*	*	Variable
20	1580[1]-020	1	+	*	*	Not tested
21	1580[1]-021	1	+	*	*	Not tested
22	1580[1]-022	3	+			Variable
23	1580[1]-023	1	+	*	*	Variable
24	1580[1]-024	1	+	*	*	Variable
25	1580[1]-025	5	+			Not tested
26	1580[1]-026	3	+			Variable
27	1580[1]-027	3	+			Low
28	1580[1]-028	4	+			Not tested
29	1580[1]-029	3	+			Variable
30	1580[1]-030	1	+	*	*	High

US 11,371,055 B2

69

TABLE 20-continued

Tobacco T <sub>0</sub> events transformed with pDAS1580 (AAD-12 (v1) + PAT)						
# Tube	Plant ID	Copy # PAT	PTU PCR AAD- 12	Full PTU and Under 2	Full PTU and 1 copy	Relative Herbicide Tolerance @
31	1580[1]-031	1	+	*	*	High
32	1580[1]-032	2	+	*		High

@ Distinguishing herbicide tolerance performance of events required assessment of relative tolerance when treated with 560 g ae/ha fluroxypyr where tolerance was variable across events.

### 10.2 Verification of High 2,4-D Tolerance in T<sub>1</sub> Tobacco.

Two to four T<sub>0</sub> individuals surviving high rates of 2,4-D and fluroxypyr were saved from each event and allowed to self fertilize in the greenhouse to give rise to T<sub>1</sub> seed. The T<sub>1</sub> seed was stratified, and sown into selection trays much like that of *Arabidopsis* (Example 7.4), followed by selective removal of untransformed nulls in this segregating population with 560 g ai/ha glufosinate (PAT) gene selection). Survivors were transferred to individual 3-inch pots in the greenhouse. These lines provided high levels of resistance to 2,4-D in the T<sub>0</sub> generation. Improved consistency of response is anticipated in T<sub>1</sub> plants not having come directly from tissue culture. These plants were compared against wildtype KY160 tobacco. All plants were sprayed with a track sprayer set at 187 L/ha. The plants were sprayed from a range of 140-2240 g ac/ha 2,4-D dimethylamine salt (DMA), 70-1120 g ace/ha triclopyr or 35-560 g ae/ha fluroxypyr. All applications were formulated in water. Each treatment was replicated 2-4 times. Plants were evaluated at 3 and 14 days after treatment. Plants were assigned injury rating with respect to stunting, chlorosis, and necrosis. The T<sub>1</sub> generation is segregating, so some variable response is expected due to difference in zygosity.

No injury was observed at 4× field rate (2240 g ae/ha) for 2,4-D or below. Some injury was observed with triclopyr treatments in one event line, but the greatest injury was observed with fluroxypyr. The fluroxypyr injury was short-lived and new growth on one event was nearly indistinguishable from the untreated control by 14 DAT (Table 21). It is important to note that untransformed tobacco is exceedingly sensitive to fluroxypyr. These results indicated commercial level 2,4-D tolerance can be provided by AAD-12 (v1), even in a very auxin-sensitive dicot crop like tobacco. These results also show resistance can be imparted to the pyridyloxyacetic acid herbicides, triclopyr and fluroxypyr. Having the ability to prescribe treatments in an herbicide tolerant crop protected by AAD-12 with various active ingredients having varying spectra of weed control is extremely useful to growers.

TABLE 21

Assessment of cross tolerance of AAD-12 (v1) T <sub>1</sub> tobacco plants' response to various phenoxy and pyridyloxy auxin herbicides.				
Herbicide	KY160- Wildtype Average %	1580(1)-004	1580(1)-018	Average % Injury of Replicates 14 DAT
		(high	(high	
		tolerance in T <sub>0</sub>	tolerance in T <sub>0</sub>	
140 g ae/ha 2,4-D DMA	45	0	0	
560 g ae/ha 2,4-D DMA	60	0	0	
2240 g ae/ha 2,4-D DMA	73	0	0	
70 g ae/ha triclopyr	40	0	5	
280 g ae/ha triclopyr	65	0	5	

70

TABLE 21-continued

Assessment of cross tolerance of AAD-12 (v1) T <sub>1</sub> tobacco plants' response to various phenoxy and pyridyloxy auxin herbicides.				
Herbicide	KY160- Wildtype Average %	1580(1)-004	1580(1)-018	Average % Injury of Replicates 14 DAT
		(high	(high	
		tolerance in T <sub>0</sub>	tolerance in T <sub>0</sub>	
1120 g ae/ha triclopyr	80	0	8	
35 g ae/ha fluroxypyr	85	0	8	
140 g ae/ha fluroxypyr	93	0	10	
560 g ae/ha fluroxypyr	100	3	18	

### 10.3 AAD-12 (v1) Heritability in Tobacco

A 100 plant progeny test was also conducted on seven T<sub>1</sub> lines of AAD-12 (v1) lines. The seeds were stratified, sown, and transplanted with respect to the procedure above with the exception that null plants were not removed by Liberty selection. All plants were then sprayed with 560 g ac/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Five out of the seven lines tested segregated as a single locus, dominant Mendelian trait (3R: 1S) as determined by Chi square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species.

### 10.4—Field Tolerance of pDAS1580 Tobacco Plants to 2,4-D, Dichloprop, Triclopyr and Fluroxypyr Herbicides.

Field level tolerance trials were conducted on three AAD-12 (v1) lines (events pDAS1580-[1]-018.001, pDAS1580-[1]-004.001 and pDAS1580-[1]-020.016) and one wild-type line (KY160) at field stations in Indiana and Mississippi. Tobacco transplants were grown in the greenhouse by planting T<sub>1</sub> seed in 72 well transplant flats (Hummert International) containing Metro 360 media according to growing conditions indicated above. The null plants were selectively removed by Liberty selection as previously described. The transplant plants were transported to the field stations and planted at either 14 or 24 inches apart using industrial vegetable planters. Drip irrigation at the Mississippi site and overhead irrigation at the Indiana site were used to keep plants growing vigorously.

The experimental design was a split plot design with 4 replications. The main plot was herbicide treatment and the sub-plot was tobacco line. The herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ac/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ac/ha and an untreated control. Plots were one row by 25-30 ft. Herbicide treatments were applied 3-4 weeks after transplanting using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual rating of injury, growth inhibition, and epinasty were taken at 7, 14 and 21 days after treatment.

AAD-12 (v1) event response to 2,4-D, triclopyr, and fluroxypyr are shown in Table 22. The non-transformed tobacco line was severely injured (63% at 14 DAT) by 2,4-D at 560 g ae/ha which is considered the 1× field application rate. The AAD-12 (v1) lines all demonstrated excellent tolerance to 2,4-D at 14 DAT with average injury of 1, 4, and 4% injury observed at the 2, 4 and 8× rates, respectively. The non-transformed tobacco line was severely injured (53% at 14 DAT) by the 2× rate of triclopyr (840 g ae/ha); whereas, AAD-12 (v1) lines demonstrated tolerance with an average of 5% injury at 14 DAT across the three lines. Fluroxypyr at 280 g ae/ha caused severe injury (99%) to the non-trans-

US 11,371,055 B2

71

formed line at 14 DAT. AAD-12 (v1) lines demonstrated increased tolerance with an average of 11% injury at 14 DAT.

These results indicate that AAD-12 (v1) transformed event lines displayed a high level of tolerance to 2,4-D, triclopyr and fluroxypyr at multiples of commercial use rates that were lethal or caused severe epinastic malformations to non-transformed tobacco under representative field conditions.

TABLE 22

AAD-12 (v1) tobacco plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.					
Herbicide Treatment		Average % Injury across locations at 14 DAT			
Active Ingredient	Rate	Wild type	pDAS1580-[1]-004.001	pDAS1580-[1]-020.016	pDAS1580-[1]-018.001
2,4-D	280 GM AE/HA	48	0	0	0
2,4-D	560 GM AE/HA	63	0	0	2
2,4-D	1120 GM AE/HA	78	1	1	2
2,4-D	2240 GM AE/HA	87	4	4	4
2,4-D	4480 GM AE/HA	92	4	4	4
Triclopyr	840 GM AE/HA	53	5	5	4
Fluroxypyr	280 GM AE/HA	99	11	11	12

#### 10.5 AAD-12 (v1) Protection Against Elevated 2,4-D Rates

Results showing AAD-12 (v1) protection against elevated rates of 2,4-D DMA in the greenhouse are shown in Table 23. T<sub>1</sub> AAD-12 (v1) plants from an event segregating 3R:1S when selected with 560 g ai/ha Liberty using the same protocol as previously described. T<sub>1</sub> AAD-1 (v3) seed was also planted for transformed tobacco controls (see PCT/US2005/014737). Untransformed KY160 was served as the sensitive control. Plants were sprayed using a track sprayer set to 187 L/ha at 140, 560, 2240, 8960, and 35840 g ae/ha 2,4-D DMA and rated 3 and 14 DAT.

AAD-12 (v1) and AA4D-1 (v3) both effectively protected tobacco against 2,4-D injury at doses up to 4× commercial use rates. AAD-12 (v1), however, clearly demonstrated a marked advantage over AAD-1 (v3) by protecting up to 64× the standard field rates.

TABLE 23

Results demonstrating protection provided by AAD-12 (v1) and AAD-1 (v3) against elevated rates of 2,4-D.			
Treatment	KY160 control	AAD-1(v3)	AAD-12 (v1)
Average % injury 14 DAT			
2240 g ae/ha 2,4-D	95	4	0
8960 g ae/ha 2,4-D	99	9	0
35840 g ae/ha 2,4-D	100	32	4

#### 10.6 Stacking of AAD-12 to Increase Herbicide Spectrum

Homozygous AAD-12 (v1) (pDAS1580) and AAD-1 (v3) (pDAB721) plants (see PCT/US2005/014737 for the latter) were both reciprocally crossed and F<sub>1</sub> seed was collected. The F<sub>1</sub> seed from two reciprocal crosses of each gene were stratified and treated 4 reps of each cross were treated under the same spray regimen as used for the other testing with one

72

of the following treatments: 70, 140, 280 g ac/ha fluroxypyr (selective for the AAD-12 (v1) gene); 280, 560, 1120 g ae/ha R-dichloroprop (selective for the AAD-1 (v3) gene); or 560, 1120, 2240 g ae/ha 2,4-D DMA (to confirm 2,4-D tolerance). Homozygous T<sub>2</sub> plants of each gene were also planted for use as controls. Plants were graded at 3 and 14 DAT. Spray results are shown in Table 24.

The results confirm that AAD-12 (v1) can be successfully stacked with AAD-1 (v3), thus increasing the spectrum herbicides that may be applied to the crop of interest (phenoxyacetic acids+phenoxypropionic acids vs phenoxyacetic acids+pyridyloxyacetic acids for AAD-1 and AAD-12, respectively). The complementary nature of herbicide cross resistance patterns allows convenient use of these two genes as complementary and stackable field-selectable markers. In crops where tolerance with a single gene may be marginal, one skilled in the art recognizes that one can increase tolerance by stacking a second tolerance gene for the same herbicide. Such can be done using the same gene with the same or different promoters; however, as observed here, stacking and tracking two complementary traits can be facilitated by the distinguishing cross protection to phenoxypropionic acids [from AAD-1 (v3)] or pyridyloxyacetic acids [AAD-12 (v1)]

TABLE 24

Comparison of auxinic herbicide cross tolerance of AAD-12 (v1) (pDAS1580) and AAD-1 (v3) (pDAB3721) T <sub>2</sub> plants compared to AAD-12 x AAD-1 F <sub>1</sub> cross and to wildtype.				
Treatment	KY160 wildtype control	AAD-12 (v1) (pDAS1580)	AAD-1(v3) (pDAB721)	AAD-12 (v1) x AAD (v3) F <sub>1</sub>
Average % injury 14 DAT				
560 g ae/ha 2,4-D	63	0	0	0
1120 g ae/ha 2,4-D	80	0	4	0
2240 g ae/ha 2,4-D	90	0	9	0
280 g ae/ha R-dichloroprop	25	15	0	0
560 g ae/ha R-dichloroprop	60	50	0	0
1120 g ae/ha R-dichloroprop	80	70	3	0
70 g ae/ha fluroxypyr	40	0	40	0
140 g ae/ha fluroxypyr	65	0	60	0
280 g ae/ha fluroxypyr	75	3	75	3

#### Example 11—Soybean Transformation

Soybean improvement via gene transfer techniques has been accomplished for such traits as herbicide tolerance (Padgett et al., 1995), amino acid modification (Falco et al., 1995), and insect resistance (Parrott et al., 1994). Introduction of foreign traits into crop species requires methods that will allow for routine production of transgenic lines using selectable marker sequences, containing simple inserts. The transgenes should be inherited as a single functional locus in order to simplify breeding. Delivery of foreign genes into cultivated soybean by microprojectile bombardment of zygotic embryo axes (McCabe et al., 1988) or somatic embryogenic cultures (Finer and McMullen, 1991), and *Agrobacterium*-mediated transformation of cotyledonary explants (Hinchey et al., 1988) or zygotic embryos (Chee et al., 1989) have been reported.

Transformants derived from *Agrobacterium*-mediated transformations tend to possess simple inserts with low copy number (Birch, 1991). There are benefits and disadvantages

US 11,371,055 B2

73

associated with each of the three target tissues investigated for gene transfer into soybean, zygotic embryonic axis (Chee et al., 1989; McCabe et al., 1988), cotyledon (Hinchee et al., 1988) and somatic embryogenic cultures (Finer and McMullen, 1991). The latter have been extensively investigated as a target tissue for direct gene transfer. Embryogenic cultures tend to be quite prolific and can be maintained over a prolonged period. However, sterility and chromosomal aberrations of the primary transformants have been associated with age of the embryogenic suspensions (Singh et al., 1998) and thus continuous initiation of new cultures appears to be necessary for soybean transformation systems utilizing this tissue. This system needs a high level of 2,4-D, 40 mg/L concentration, to initiate the embryogenic callus and this poses a fundamental problem in using the AAD-12 (v1) gene since the transformed locus could not be developed further with 2,4-D in the medium. So, the meristem based transformation is ideal for the development of 2,4-D resistant plant using AAD-12 (v1).

#### 11.1 Gateway Cloning of Binary Constructs

The AAD-12 (v1) coding sequence was cloned into five different Gateway Donor vectors containing different plant promoters. The resulting AAD-12 (v1) plant expression cassettes were subsequently cloned into a Gateway Destination Binary vector via the LR Clonase reaction (Invitrogen Corporation, Carlsbad Ca, Cat #11791-019).

An NcoI-SacI fragment containing the AAD-12 (v1) coding sequence was digested from DASPICO12 and ligated into corresponding NcoI-SacI restriction sites within the following Gateway Donor vectors: pDAB3912 (attL1//CsVMV promoter//AtuORF23 3'UTR//attL2); pDAB3916 (attL1//AtUbi10 promoter//AtuORF23 3'UTR//attL2); pDAB4458 (attL1//AtUbi3 promoter//AtuORF23 3'UTR//attL2); pDAB4459 (attL1//ZmUbi1 promoter//AtuORF23 3'UTR//attL2); and pDAB4460 (attL1//AtAct2 promoter//AtuORF23 3'UTR//attL2). The resulting constructs containing the following plant expression cassettes were designated: pDAB4463 (attL1//CsVMV promoter//AAD-12 (v1)//AtuORF23 3'UTR 1//attL2); pDAB4467 (attL1//AtUbi10 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4471 (attL1//AtUbi3 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); pDAB4475 (attL1//ZmUbi1 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2); and pDAB4479 (attL1//AtAct2 promoter//AAD-12 (v1)//AtuORF23 3'UTR//attL2). These constructs were confirmed via restriction enzyme digestion and sequencing.

The plant expression cassettes were recombined into the Gateway Destination Binary vector pDAB4484 (RB7 MARv3//attR1-ccdB-chloramphenicol resistance-attR2//CsVMV promoter//PATv6//AtuORF1 3'UTR) via the Gateway LR Clonase reaction. Gateway Technology uses lambda phage-based site-specific recombination instead of restriction endonuclease and ligase to insert a gene of interest into an expression vector. Invitrogen Corporation, Gateway Technology: A Universal Technology to Clone DNA Sequences for Functional Analysis and Expression in multiple Systems, Technical Manual, Catalog #s 12535-019 and 12535-027, Gateway Technology Version E, Sep. 22, 2003. #25-022. The DNA recombination sequences (attL, and attR), and the LR Clonase enzyme mixture allows any DNA fragment flanked by a recombination site to be transferred into any vector containing a corresponding site. The attL1 site of the donor vector corresponds with attR1 of the binary vector. Likewise, the attL2 site of the donor vector corresponds with attR2 of the binary vector. Using the Gateway Technology the plant expression cassette (from the donor vector) which is flanked by the attL sites can be

74

recombined into the attR sites of the binary vector. The resulting constructs containing the following plant expression cassettes were labeled as: pDAB4464 (RB7 MARv3//CsVMV promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4468 (RB7 MARv3//AtUbi10 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4472 (RB7 MARv3//AtUbi3 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); pDAB4476 (RB7 MARv3//ZmUbi1 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR); and pDAB4480 (RB7 MARv3//AtAct2 promoter//AAD-12 (v1)//AtuORF23 3'UTR//CsVMV promoter//PATv6//AtuORF1 3'UTR) (see Table 8). These constructs were confirmed via restriction enzyme digestion and sequencing.

#### 11.2 Transformation Method 1: Cotyledonary Node Transformation of Soybean Mediated by *Agrobacterium tumefaciens*.

The first reports of soybean transformation targeted meristematic cells in the cotyledonary node region (Hinchee et al., 1988) and shoot multiplication from apical meristems (McCabe et al., 1988). In the *A. tumefaciens*-based cotyledonary node method, explant preparation and culture media composition stimulate proliferation of auxiliary meristems in the node (Hinchee et al., 1988). It remains unclear whether a truly dedifferentiated, but totipotent, callus culture is initiated by these treatments. The recovery of multiple clones of a transformation event from a single explant and the infrequent recovery of chimeric plants (Clemente et al., 2000; Olhoft et al., 2003) indicates a single cell origin followed by multiplication of the transgenic cell to produce either a proliferating transgenic meristem culture or a uniformly transformed shoot that undergoes further shoot multiplication. The soybean shoot multiplication method, originally based on microprojectile bombardment (McCabe et al., 1988) and, more recently, adapted for *Agrobacterium*-mediated transformation (Martinell et al., 2002), apparently does not undergo the same level or type of dedifferentiation as the cotyledonary node method because the system is based on successful identification of germ line chimeras. The range of genotypes that have been transformed via the *Agrobacterium*-based cotyledonary node method is steadily growing (Olhoft and Somers, 2001). This de novo meristem and shoot multiplication method is less limited to specific genotypes. Also, this is a non 2,4-D based protocol which would be ideal for 2,4-D selection system. Thus, the cotyledonary node method may be the method of choice to develop 2,4-D resistant soybean cultivars. Though this method was described as early as 1988 (Hinchee et al., 1988), only very recently has it been optimized for routine high frequency transformation of several soybean genotypes (Zhang et al., 1999; Zeng et al., 2004).

##### 11.2.1—Plant Transformation Production of AAD-12 (v1) Tolerant Phenotypes.

Seed derived explants of "Maverick" and the *Agrobacterium* mediated cot-node transformation protocol was used to produce AAD-12 (v1) transgenic plants.

##### 11.2.2—*Agrobacterium* Preparation and Inoculation

*Agrobacterium* strain EHA101 (Hood et al. 1986), carrying each of five binary pDAB vectors (Table 8) was used to initiate transformation. Each binary vector contains the AAD-12 (v1) gene and a plant-selectable gene (PAT) cassette within the T-DNA region. Each gene is driven by the promoters listed in Table 8 and these plasmids were mobilized into the EHA101 strain of *Agrobacterium* by electroporation. The selected colonies were then analyzed for the



US 11,371,055 B2

75

integration of genes before the *Agrobacterium* treatment of the soybean explants. Maverick seeds were used in all transformation experiments and the seeds were obtained from University of Missouri, Columbia, Mo.

*Agrobacterium*-mediated transformation of soybean (*Glycine max*) using the PAT gene as a selectable marker coupled with the herbicide glufosinate as a selective agent was carried out followed a modified procedure of Zeng et al. (2004). The seeds were germinated on B5 basal medium (Gamborg et al. 1968) solidified with 3 g/L Phytigel (Sigma-Aldrich, St. Louis, Mo.); added 1-cysteine to the co-cultivation medium at 400 mg/L and co-cultivation lasted 5 days (Olhoft and Somers 2001); shoot initiation, shoot elongation, and rooting media were supplemented with 50 mg/L cefotaxime, 50 mg/L timentin, 50 mg/L vancomycin, and solidified with 3 g/L Phytigel. Selected shoots were then transferred to the rooting medium. The optimal selection scheme was the use of glufosinate at 8 mg/L across the first and second shoot initiation stages in the medium and 3-4 mg/L during shoot elongation in the medium.

Prior to transferring elongated shoots (3-5 cm) to rooting medium, the excised end of the internodes were dipped in 1 mg/L indole 3-butyric acid for 1-3 min to promote rooting (Khan et al. 1994). The shoots struck roots in 25×100 mm glass culture tubes containing rooting medium and then they were transferred to soil mix for acclimatization of plantlets in Metro-mix 200 (Hummert International, Earth City, Mo.) in open Magenta boxes in Conviron. Glufosinate, the active ingredient of Liberty herbicide (Bayer Crop Science), was used for selection during shoot initiation and elongation. The rooted plantlets were acclimated in open Magenta boxes for several weeks before they were screened and transferred to the greenhouse for further acclimation and establishment.

11.2.3—Assay of Putatively Transformed Plantlets, and Analyses Established  $T_0$  Plants in the Greenhouse.

The terminal leaflets of selected leaves of these plantlets were leaf painted with 50 mg/L of glufosinate twice with a week interval to observe the results to screen for putative transformants. The screened plantlets were then transferred to the greenhouse and after acclimation the leaves were painted with glufosinate again to confirm the tolerance status of these plantlets in the GH and deemed to be putative transformants.

Plants that are transferred to the greenhouse can be assayed for the presence of an active PAT gene further with a non-destructive manner by painting a section of leaf of the  $T_0$  primary transformant, or progeny thereof, with a glufosinate solution [0.05-2% v/v Liberty Herbicide, preferably 0.25-1.0% (v/v),=500-2000 ppm glufosinate, Bayer Crop Science]. Depending on the concentration used, assessment for glufosinate injury can be made 1-7 days after treatment. Plants can also be tested for 2,4-D tolerance in a non-destructive manner by selective application of a 2,4-D solution in water (0.25-1% v/v commercial 2,4-D dimeth-

76

ylamine salt formulation, preferably 0.5% v/v=2280 ppm 2,4-D ae) to the terminal leaflet of the newly expanding trifoliolate one or two, preferably two, nodes below the youngest emerging trifoliolate. This assay allows assessment of 2,4-D sensitive plants 6 hours to several days after application by assessment of leaf flipping or rotation >90 degrees from the plane of the adjacent leaflets. Plants tolerant to 2,4-D will not respond to 2,4-D. To plants will be allowed to self fertilize in the greenhouse to give rise to  $T_1$  seed.  $T_1$  plants (and to the extent enough  $T_0$  plant clones are produced) will be sprayed with a range of herbicide doses to determine the level of herbicide protection afforded by AAD-12 (v1) and PAT genes in transgenic soybean. Rates of 2,4-D used on  $T_0$  plants will typically comprise one or two selective rates in the range of 100-1120 g ae/ha using a track sprayer as previously described.  $T_1$  plants will be treated with a wider herbicide dose ranging from 50-3200 g ae/ha 2,4-D. Likewise,  $T_0$  and  $T_1$  plants can be screened for glufosinate resistance by postemergence treatment with 200-800 and 50-3200 g ae/ha glufosinate, respectively. Glyphosate resistance (in plants transformed with constructs that contain EPSPS) or another glyphosate tolerance gene can be assessed in the  $T_1$  generation by postemergence applications of glyphosate with a dose range from 280-2240 g ae/ha glyphosate. Analysis of protein expression will occur as described in below. Individual  $T_0$  plants were assessed for the presence of the coding region of the gene of interest (AAD-12 (v1) or PAT v6) and copy number. Determination of the inheritance of AAD-12 (v1) will be made using  $T_1$  and  $T_2$  progeny segregation with respect to herbicide tolerance as described in previous examples.

A subset of the initial transformants were assessed in the  $T_0$  generation according to the methods above. Any plant confirmed as having the AAD-12 (v1) coding region, regardless of the promoter driving the gene did not respond to the 2,4-D leaf painting whereas wildtype Maverick soybeans did (Table Sec 11.2.3). PAT-only transformed plants responded the same at wildtype plants to leaf paint applications of 2,4-D

2,4-D was applied to a subset of the plants that were of similar size to the wildtype control plants with either 560 or 1120 g ac 2,4-D. All AAD-12 (v1)-containing plants were clearly resistant to the herbicide application versus the wildtype Maverick soybeans. A slight level of injury (2 DAT) was observed for two AAD-12 (v1) plants, however, injury was temporary and no injury was observed 7 DAT. Wildtype control plants were severely injured 7-14 DAT at 560 g ae/ha 2,4-D and killed at 1120 g ae/ha. These data are consistent with the fact that AAD-12 (v1) can impart high tolerance (>2× field rates) to a sensitive crop like soybeans. The screened plants were then sampled for molecular and biochemical analyses for the confirmation of the AAD12 (v1) genes integration, copy number, and their gene expression levels as described below and reported in Table 25.

TABLE 25

$T_0$  soybean response to 2,4-D leaf paint and 2,4-D spray application.

Chloroplast responses to 2,4-D leaf priming and 2,4-D spray application														
Leaf flip assay														
NODE 2,4-D @ (18 HAT)														
Construct (pDAB#)	Gene	Promoter	Event	LEAF PAINTED	Node N-1	N-2	Spray POST over the top with 2,4-D (g ae/ha)	Stage at appl (# nodes)	ELISA (ng/mL)	South- em Copy number	PCR coding region	% injury 2 DAT	% injury 7 DAT	% injury 14 DAT
4464	AAD-12	CsVMV	D-1-14	N-1	0		0	>10	5246.83	2	+	X	X	0
4464	AAD-12	CsVMV	D-2-9	N-2		0	0	>10	204.27	1	+	X	X	0

## US 11,371,055 B2

77

78

TABLE 25-continued

T <sub>0</sub> soybean response to 2,4-D leaf paint and 2,4-D spray application.														
Leaf flip assay NODE 2,4-D @ (18 HAT)														
Construct (pDAB#)	Gene	Promoter	Event	LEAF PAINTED	Node N-1	N-2	Spray POST over the top with 2,4-D (g ae/ha)	Stage at appl (# nodes)	ELISA] (ng/mL)	South- ern Copy number	PCR coding region	% injury 2 DAT	% injury 7 DAT	% injury 14 DAT
4468	AAD-12	AtUbi10	D-3-7	N-2		0	0	>10	4.65	1	+	0	0	0
4468	AAD-12	AtUbi10	D-4-11B	N-2		0	0	8	1452.84	2	+	0	0	0
4468	AAD-12	AtUbi10	D-4-16	N-2		0	0	>10	653.21	2	+	X	X	0
4480	AAD-12	AtAct2	D-9-1	N-2		0	0	>10	248.33	3 or 4	+	X	X	0
4464	AAD-12	CsVMV	D-2-14	N-2		0	560	7	4917.43	2	+	0	0	0
4468	AAD-12	AtUbi10	D-3-5	N-2		0	560	8	365.75	1	+	0	0	0
4468	AAD-12	AtUbi10	D-3-6	N-1	0		560	5	714.79	3	+	0	0	0
4472	AAD-12	AtUbi3	D-5-2	N-1	0		560	6	0.58	1	+	5	0	0
4468	AAD-12	AtUbi10	D-3-9	N-2		0	1120	6	2657.26	3	+	0	0	0
4468	AAD-12	AtUbi10	D-4-17	N-2		0	1120	7	286.14	5	+	5	0	0
4499	PAT	CsVMV	D-2-3	N-2	1		0	>10	2.36	5	+	X	X	0
Maverick	WT		WT-10	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-2	NT		1	ND	ND	ND	ND	ND	ND	ND	ND
Maverick	WT		WT-3	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-4	NT			0	4	ND	ND	ND	0	0	0
Maverick	WT		WT-5	NT			560	4	ND	ND	ND	50	60	60
Maverick	WT		WT-6	NT			560	4	ND	ND	ND	70	90	80
Maverick	WT		WT-7	NT			560	4	ND	ND	ND	70	80	80
Maverick	WT		WT-10	NT			1120	4	ND	ND	ND	70	90	100
Maverick	WT		WT-8	NT			1120	4	ND	ND	ND	70	95	100
Maverick	WT		WT-9	NT			1120	4	ND	ND	ND	70	95	100

1 = Flip

0 = No Flip

ND = Not determined

## 11.2.4—Molecular Analyses: Soybean

11.2.4.1—Tissue Harvesting DNA Isolation and Quantification. Fresh tissue is placed into tubes and lyophilized at 4° C. for 2 days. After the tissue is fully dried, a tungsten bead (Valenite) is placed in the tube and the samples are subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure is then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA is then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μL.

11.2.4.2—Polymerase Chain Reaction. A total of 100 ng of total DNA is used as the template. 20 mM of each primer is used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for the AAD-12 (v1) PTU are (Forward—ATAATGCCAGC CTGTTAAACGCC) (SEQ ID NO:8) and (Reverse—CTCAAGCATATGAATGACCT CGA) (SEQ ID NO:9). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 63° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. Primers for Coding Region PCR AAD-12 (v1) are (Forward—ATGGCTCATGCTGCCCTCAGCC) (SEQ ID NO:10) and (Reverse—CGGGC AGGCCTAACTCCAC-CAA) (SEQ ID NO: 11). The PCR reaction is carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 1 minute and 45 seconds followed by 72° C. for 10 minutes. PCR products are analyzed by electrophoresis on a 1% agarose gel stained with EtBr.

11.2.4.3—Southern Blot Analysis. Southern blot analysis is performed with total DNA obtained from Qiagen DNeasy kit. A total of 10 μg of genomic DNA is subjected to an

overnight digestion to obtain integration data. After the overnight digestion an aliquot of ~100 ng is run on a 1% gel to ensure complete digestion. After this assurance the samples are run on a large 0.85% agarose gel overnight at 40 volts. The gel is then denatured in 0.2 M NaOH, 0.6 M NaCl for 30 minutes. The gel is then neutralized in 0.5 M Tris HCl, 1.5 M NaCl pH of 7.5 for 30 minutes. A gel apparatus containing 20×SSC is then set up to obtain a gravity gel to nylon membrane (Millipore INYC00010) transfer overnight. After the overnight transfer the membrane is then subjected to UV light via a crosslinker (Stratagene UV stratalinker 1800) at 1200×100 microjoules. The membrane is then washed in 0.1% SDS, 0.1 SSC for 45 minutes. After the 45 minute wash, the membrane is baked for 3 hours at 80° C. and then stored at 4° C. until hybridization. The hybridization template fragment is prepared using the above coding region PCR using plasmid DNA. The product is run on a 1% agarose gel and excised and then gel extracted using the Qiagen (28706) gel extraction procedure. The membrane is then subjected to a pre-hybridization at 60° C. step for 1 hour in Perfect Hyb buffer (Sigma H7033). The Prime it RmT dCTP-labeling rxn (Stratagene 300392) procedure is used to develop the p32 based probe (Perkin Elmer). The probe is cleaned up using the Probe Quant. G50 columns (Amersham 27-5335-01). Two million counts CPM are used to hybridize the southern blots overnight. After the overnight hybridization the blots are then subjected to two 20 minute washes at 65° C. in 0.1% SDS, 0.1 SSC. The blots are then exposed to film overnight, incubating at -80° C.

## 11.2.5—Biochemical Analyses: Soybean

11.2.5.1—Tissue Sampling and Extracting AAD-12 (v1) Protein from Soybean Leaves. Approximately 50 to 100 mg of leaf tissue was sampled from the N-2 leaves that were 2,4-D leaf painted, but after 1 DAT. The terminal N-2 leaflet was removed and either cut into small pieces or 2-single-



US 11,371,055 B2

79

hole-punched leaf discs (~0.5 cm in diameter) and were frozen on dry ice instantly. Further protein analysis (ELISA and Western analysis) was completed according to methods described in Example 9.

11.2.6— $T_1$  Progeny Evaluation.  $T_0$  plants will be allowed to self fertilize to derive  $T_1$  families. Progeny testing (segregation analysis) will be assayed using glufosinate at 560 g ai/ha as the selection agent applied at the V1-V2 growth stage. Surviving plants will be further assayed for 2,4-D tolerance at one or more growth stages from V2-V6. Seed will be produced through self fertilization to allow broader herbicide testing on the transgenic soybean.

AAD-12 (v1) transgenic Maverick soybean plants have been generated through *Agrobacterium*-mediated cot-node transformation system. The  $T_0$  plants obtained tolerated up to 2× levels of 2,4-D field applications and developed fertile seeds. The frequency of fertile transgenic soybean plants was up to 5.9%. The integration of the AAD1-12 (v1) gene into the soybean genome was confirmed by Southern blot analysis. This analysis indicated that most of the transgenic plants contained a low copy number. The plants screened with AAD-12 (v1) antibodies showed positive for ELISA and the appropriate band in Western analysis.

11.3 Transformation Method 2: Aerosol-Beam Mediated Transformation of Embryogenic Soybean Callus Tissue.

Culture of embryogenic soybean callus tissue and subsequent beaming can be accomplished as described in U.S. Pat. No. 6,809,232 (Held et al.) to create transformants using one or more constructs in Table 8.

11.4 Transformation Method 3. Biolistic Bombardment of Soybean

This can be accomplished using mature seed derived embryonic axes meristem (McCabe et al. (1988)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

11.5 Transformation Method 4. Whiskers Mediated Transformation.

Whisker preparation and whisker transformation can be anticipated according to methods described previously by Terakawa et al. (2005)). Following established methods of biolistic bombardment, one can expect recovery of transformed soybean plants. Once plants are regenerated and evaluation of events could occur as described in Example 11.2.

Maverick seeds were surface-sterilized in 70% ethanol for 1 min followed by immersion in 1% sodium hypochlorite for 20 min. and then rinsed three times in sterile distilled water. The seeds were soaked in distilled water for 18-20 h. The embryonic axes were excised from seeds, and the apical meristems were exposed by removing the primary leaves. The embryonic axes were positioned in the bombardment medium [BM: MS (Murashige and Skoog 1962) basal salts medium, 3% sucrose and 0.8% phytagel Sigma, pH 5.7] with the apical region directed upwards in 5-cm culture dishes containing 12 ml culture medium.

11.6 Transformation Method 5. Particle bombardment-mediated transformation for embryogenic callus tissue can be optimized for according to previous methods (Khalafalla et al., 2005; El-Shemy et al., 2004, 2006). Regenerated plants can also be assessed according to Example 11.2.

Example 12-AAD-12 (v1) in Cotton

12.1—Cotton Transformation Protocol.

Cotton seeds (Co310 genotype) are surface-sterilized in 95% ethanol for 1 minute, rinsed, sterilized with 50%

80

commercial bleach for twenty minutes, and then rinsed 3 times with sterile distilled water before being germinated on G-media (Table 26) in Magenta GA-7 vessels and maintained under high light intensity of 40-60  $\mu$ E/m<sup>2</sup>, with the photoperiod set at 16 hours of light and 8 hours dark at 28° C.

Cotyledon segments (~5 mm) square are isolated from 7-10 day old seedlings into liquid M liquid media (Table 26) in Petri plates (Nunc, item #0875728). Cut segments are treated with an *Agrobacterium* solution (for 30 minutes) then transferred to semi-solid M-media (Table 26) and undergo co-cultivation for 2-3 days. Following co-cultivation, segments are transferred to MG media (Table 26). Carbenicillin is the antibiotic used to kill the *Agrobacterium* and glufosinate-ammonium is the selection agent that would allow growth of only those cells that contain the transferred gene.

*Agrobacterium* Preparation. Inoculate 35 ml of Y media (Table 26) (containing streptomycin (100 mg/ml stock) and erythromycin (100 mg/ml stock)), with one loop of bacteria to grow overnight in the dark at 28° C., while shaking at 150 rpm. The next day, pour the *Agrobacterium* solution into a sterile oakridge tube (Nalge-Nunc, 3139-0050), and centrifuge for in Beckman J2-21 at 8,000 rpm for 5 minutes. Pour off the supernatant and resuspend the pellet in 25 ml of M liquid (Table 26) and vortex. Place an aliquot into a glass culture tube (Fisher, 14-961-27) for Klett reading (Klett-Summerson, model 800-3). Dilute the new suspension using M liquid media to a Klett-meter reading of 10<sup>8</sup> colony forming units per ml with a total volume of 40 ml.

After three weeks, callus from the cotyledon segments is isolated and transferred to fresh MG media. The callus is transferred for an additional 3 weeks on MG media. In a side-by-side comparison. MG media can be supplemented with dichlorprop (added to the media at a concentration of 0.01 and 0.05 mg/L) to supplement for the degradation of the 2,4-D, since dichlorprop is not a substrate for the AAD-12 enzyme, however dichlorprop is more active on cotton than 2,4-D. In a separate comparison, segments which were plated on MG media containing no growth regulator compared to standard MG media, showed reduced callusing, but there still is callus growth. Callus is then transferred to CG-media (Table 26), and transferred again to fresh selection medium after three weeks. After another three weeks the callus tissue is transferred to D media (Table 26) lacking plant growth regulators for embryogenic callus induction. After 4-8 weeks on this media, embryogenic callus is formed, and can be distinguished from the non-embryogenic callus by its yellowish-white color and granular cells. Embryos start to regenerate soon after and are distinct green in color. Cotton can take time to regenerate and form embryos, one of the ways to speed up this process is to stress the tissue. Desiccation is a common way to accomplish this, via changes in the microenvironment of the tissue and plate, by using less culture media and/or adopting various modes of plate enclosure (taping versus parafilm).

Larger, well-developed embryos are isolated and transferred to DK media (Table 26) for embryo development. After 3 weeks (or when the embryos have developed), germinated embryos are transferred to fresh media for shoot and root development. After 4-8 weeks, any well-developed plants are transferred into soil and grown to maturity. Following a couple of months, the plant has grown to a point that it can be sprayed to determine if it has resistance to 2,4-D.

US 11,371,055 B2

81

82

TABLE 26

Media for Cotton Transformation								
Ingredients in 1 liter	G	M liquid	M	MG	CG	D	DK	Y
LS Salts (5X)	200 ml	200 ml	200 ml	200 ml	200 ml			
Glucose		30 grams	30 grams	30 grams	30 grams	20 grams		
modified B5 vit (1000x)	1 ml	1 ml	1 ml	1 ml	1 ml	10 ml	1 ml	
kinetin (1 mM)		1 ml	1 ml	1 ml	4.6 ml		0.5 ml	
2,4-D (1 mM)		1 ml	1 ml	1 ml				
Agar	8 grams		8 grams	8 grams	8 grams	8 grams	8 grams	
DKW salts (D190)						1 package	1 package	
MYO-Inositol (100x)						1 ml	10 ml	
Sucrose 3%	30 grams						30 grams	10 grams
NAA								
Carbenicillin (250 mg/ml)				2 ml	0.4 ml			
GLA (10 mg/ml)				0.5 ml	0.3 ml			
Peptone								10 grams
Yeast Extract								10 grams
NaCl								5 grams

### 12.2—Cell Transformation.

Several experiments were initiated in which cotyledon segments were treated with *Agrobacterium* containing pDAB724. Over 2000 of the resulting segments were treated using various auxin options for the proliferation of pDAB724 cotton callus, either: 0.1 or 0.5 mg/L R-dichloroprop, standard 2,4-D concentration and no auxin treatment. The callus was selected on glufosinate-ammonium, due to the inclusion of the PAT gene in the construct. Callus line analysis in the form of PCR and Invader will be used to determine if and to be sure the gene was present at the callus stage: then callus lines that are embryogenic will be sent for Western analysis, essentially as described in section 11.2.3. Embryogenic cotton callus was stressed using desiccation techniques to improve the quality and quantity of the tissue recovered.

Almost 200 callus events have been screened for intact PTU and expression using Western analysis for the AAD-12 (v1) gene. Below is a subset of the data for some of the cotton callus that has been tested.

Construct	Line Number	AAD-12 PTU	AAD-12 Invader	AAD-12 ng/ml
pDAB724	1	+	+	79.89
pDAB724	2	+	+	17.34
pDAB724	3	+	+	544.80
pDAB724	4	+	+	32.63
pDAB724	5	+	+	82.77
pDAB724	83	+	+	795.50
pDAB724	84	+	+	613.35
pDAB724	85	+	+	1077.75
pDAB724	86	+	+	437.74
pDAB724	87	+	+	286.51
pDAB724	88	+	+	517.59
pDAB724	89	+	+	1250.70

### 12.3—Plant Regeneration.

AAD-12 (v1) cotton lines that have produced plants according to the above protocol will be sent to the greenhouse. To demonstrate the AAD-12 (v1) gene provides resistance to 2,4-D in cotton, both the AAD-12 (v1) cotton plant and wild-type cotton plants will be sprayed with a track sprayer delivering 560 g ae/ha 2,4-D at a spray volume of 187 L/ha. The plants will be evaluated at 3 and 14 days after treatment. Plants surviving a selective rate of 2,4-D will be self pollinated to create T<sub>1</sub> seed or outcrossed with an elite

cotton line to produce F<sub>1</sub> seed. The subsequent seed produced will be planted and evaluated for herbicide resistance as previously described. AAD-12 (v1) events can be combined with other desired HT or IR traits as described in experiments 18, 19, 22, and 23.

### Example 13—*Agrobacterium* Transformation of Other Crops

In light of the subject disclosure, additional crops can be transformed according to the subject invention using techniques that are known in the art. For *Agrobacterium*-mediated transformation of rye, see, e.g., Popelka and Altpeter (2003). For *Agrobacterium*-mediated transformation of soybean, see, e.g., Hinchey et al., 1988. For *Agrobacterium*-mediated transformation of sorghum, see, e.g., Zhao et al., 2000. For *Agrobacterium*-mediated transformation of barley, see, e.g., Tingay et al., 1997. For *Agrobacterium*-mediated transformation of wheat, see, e.g., Cheng et al., 1997. For *Agrobacterium*-mediated transformation of rice, see, e.g., Hiei et al., 1997.

The Latin names for these and other plants are given below. It should be clear that these and other (non-*Agrobacterium*) transformation techniques can be used to transform AAD-12 (v1), for example, into these and other plants, including but not limited to Maize (*Zea mays*), Wheat (*Triticum* spp.), Rice (*Oryza* spp. and *Zizania* spp.), Barley (*Hordeum* spp.), Cotton (*Abroma augusta* and *Gossypium* spp.), Soybean (*Glycine max*), Sugar and table beets (*Beta* spp.), Sugar cane (*Arenga pinnata*), Tomato (*Lycopersicon esculentum* and other spp., *Physalis ixocarpa*, *Solanum incanum* and other spp., and *Cyphomandra betacea*). Potato (*Solanum tuberosum*), Sweet potato (*Ipomoea batatas*), Rye (*Secale* spp.), Peppers (*Capsicum annuum*, *sinense*, and *frutescens*), Lettuce (*Lactuca sativa*, *perennis*, and *pulchella*), Cabbage (*Brassica* spp). Celery (*Apium graveolens*), Eggplant (*Solanum melongena*), Peanut (*Arachis hypogea*). Sorghum (all *Sorghum* species), Alfalfa (*Medicago sativa*), Carrot (*Daucus carota*), Beans (*Phaseolus* spp. and other genera), Oats (*Avena sativa* and *strigosa*), Peas (*Pisum*, *Vigna*, and *Tetragonolobus* spp.), Sunflower (*Helianthus annuus*), Squash (*Cucurbita* spp.), Cucumber (*Cucumis sativa*), Tobacco (*Nicotiana* spp.). *Arabidopsis* (*Arabidopsis thaliana*). Turfgrass (*Lolium*, *Agrostis*, *Poa*, *Cynodon*, and other genera), Clover (*Trifolium*), Vetch (*Vi-*

US 11,371,055 B2

83

*cia*). Such plants, with AAD-12 (v1) genes, for example, are included in the subject invention.

AAD-12 (v1) has the potential to increase the applicability of key auxinic herbicides for in-season use in many deciduous and evergreen timber cropping systems. Triclopyr, 2,4-D, and/or fluroxypyr resistant timber species would increase the flexibility of over-the-top use of these herbicides without injury concerns. These species would include, but not limited to: Alder (*Alnus* spp.), ash (*Fraxinus* spp.), aspen and poplar species (*Populus* spp.), beech (*Fagus* spp.), birch (*Betula* spp.), cherry (*Prunus* spp.), eucalyptus (*Eucalyptus* spp.), hickory (*Carya* spp.), maple (*Acer* spp.), oak (*Quercus* spp.), and pine (*Pinus* spp.). Use of auxin resistance for the selective weed control in ornamental and fruit-bearing species is also within the scope of this invention. Examples could include, but not be limited to, rose (*Rosa* spp.), burning bush (*Euonymus* spp.), *petunia* (*Petunia* spp.), *begonia* (*Begonia* spp.), *rhododendron* (*Rhododendron* spp.), crabapple or apple (*Malus* spp.), pear (*Pyrus* spp.), peach (*Prunus* spp.), and marigolds (*Tagetes* spp.).

#### Example 14—Further Evidence of Surprising Results: AAD-12 vs. AAD-2

##### 14.1—AAD-2 (v1) Initial Cloning

Another gene was identified from the NCBI database (see the ncbi.nlm.nih.gov website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as AAD-2 (v1) for consistency. Percent identity was determined by first translating both the AAD-2 and *tfdA* DNA sequences (SEQ ID NO:12 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

The strain of *Bradyrhizobium japonicum* containing the AAD-2 (v1) gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at  $-80^{\circ}\text{C}$ . in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at  $28^{\circ}\text{C}$ . for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at  $28^{\circ}\text{C}$ . on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward (SEQ ID NO:16): 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse (SEQ ID NO:17): 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 of PCT/US2005/014737 (added a Xho I site)].

Fifty microliter reactions were set up as follows: Fail Safe Buffer 25  $\mu\text{l}$ , ea. primer 1  $\mu\text{l}$  (50 ng/ $\mu\text{l}$ ), gDNA 1  $\mu\text{l}$  (200 ng/ $\mu\text{l}$ ),  $\text{H}_2\text{O}$  21  $\mu\text{l}$ , Taq polymerase 1  $\mu\text{l}$  (2.5 units/ $\mu\text{l}$ ). Three Fail Safe Buffers-A, B, and C-were used in three separate reactions. PCR was then carried out under the following conditions:  $95^{\circ}\text{C}$ . 3.0 minutes heat denature cycle;  $95^{\circ}\text{C}$ . 1.0 minute,  $50^{\circ}\text{C}$ . 1.0 minute,  $72^{\circ}\text{C}$ . 1.5 minutes, for 30 cycles; followed by a final cycle of  $72^{\circ}\text{C}$ . 5 minutes, using the FailSafe PCR System (Epicenter cat. #FS99100). The resulting  $\sim 1$  kb PCR product was cloned into pCR 2.1

84

(Invitrogen cat. #K4550-40) following the included protocol, with chemically competent TOP10F' *E. coli* as the host strain, for verification of nucleotide sequence.

Ten of the resulting white colonies were picked into 3  $\mu\text{l}$  Luria Broth+1000  $\mu\text{g}/\text{ml}$  Ampicillin (LB Amp), and grown overnight at  $37^{\circ}\text{C}$ . with agitation. Plasmids were purified from each culture using Nucleospin Plus Plasmid Miniprep Kit (BD Biosciences cat. #K3063-2) and following included protocol. Restriction digestion of the isolated DNA's was completed to confirm the presence of the PCR product in the pCR2.1 vector. Plasmid DNA was digested with the restriction enzyme EcoRI (New England Biolabs cat. #R0101S). Sequencing was carried out with Beckman CEQ Quick Start Kit (Beckman Coulter cat. #608120) using M13 Forward [5' GTA AAA CGA CGG CCA G 3'] (SEQ ID NO:6) and Reverse [5' CAG GAA ACA GCT ATG AC 3'] (SEQ ID NO:7) primers, per manufacturers instructions. This gene sequence and its corresponding protein was given a new general designation AAD-2 (v1) for internal consistency.

##### 14.2—Completion of AAD-2 (v1) Binary Vector.

The AAD-2 (v1) gene was PCR amplified from pDAB3202. During the PCR reaction alterations were made within the primers to introduce the AflIII and SacI restriction sites in the 5' primer and 3' primer, respectively. See PCT/US2005/014737. The primers "NcoI of Brady" [5' TAT ACC ACA TGT CGA TCG CCA TCC GGC AGC TT 3'] (SEQ ID NO:14) and "SacI of Brady" [5' GAG CTC CTA TCA CTC CGC CGC CTG CTG CTG CAC 3'] (SEQ ID NO:15) were used to amplify a DNA fragment using the Fail Safe PCR System (Epicentre). The PCR product was cloned into the pCR2.1 TOPO TA cloning vector (Invitrogen) and sequence verified with M13 Forward and M13 Reverse primers using the Beckman Coulter "Dye Terminator Cycle Sequencing with Quick Start Kit" sequencing reagents. Sequence data identified a clone with the correct sequence (pDAB716). The AflIII/SacI AAD-2 (v1) gene fragment was then cloned into the NcoI/SacI pDAB726 vector. The resulting construct (pDAB717); AtUbi10 promoter: Nt OSM 5'UTR: AAD-2 (v1); Nt OSM3'UTR: ORF1 polyA 3'UTR was verified with restriction digests (with NcoI/SacI). This construct was cloned into the binary pDAB3038 as a NotI-NotI DNA fragment. The resulting construct (pDAB767); AtUbi10 promoter: Nt OSM5'UTR: AAD-2 (v1); Nt OSM 3'UTR: ORF1 polyA 3'UTR: CsVMV promoter. PAT: ORF25/26 3'UTR was restriction digested (with NotI, EcoRI, HindIII, NcoI, PvuII, and SalI) for verification of the correct orientation. The completed construct (pDAB767) was then used for transformation into *Agrobacterium*.

##### 14.3—Evaluation of Transformed *Arabidopsis*.

Freshly harvested  $T_1$  seed transformed with a plant optimized AAD-12 (v1) or native AAD-2 (v1) gene were planted and selected for resistance to glufosinate as previously described. Plants were then randomly assigned to various rates of 2,4-D (50-3200 g ae/ha). Herbicide applications were applied by track sprayer in a 187 L/ha spray volume. 2,4-D used was the commercial dimethylamine salt formulation (456 g ae/L, NuFarm, St Joseph, Mo.) mixed in 200 mM Tris buffer (pH 9.0) or 200 mM HEPES buffer (pH7.5).

AAD-12 (v1) and AAD-2 (v1) did provide detectable 2,4-D resistance versus the transformed and untransformed control lines: however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual  $T_1$  *Arabidopsis* plants. Surprisingly, AAD-2 (v1) and AAD-2 (v2) transformants were far less resistant to 2,4-D than the AAD-12 (v1) gene, both from a frequency of highly tolerant plants as well as overall average injury. No plants



US 11,371,055 B2

85

transformed with AAD-2 (v1) survived 200 g ac/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (see PCT/US2005/014737). Conversely, AAD-12 (v1) had a population injury average of about 6% when treated with 3,200 g ac/ha 2,4-D (Table 11). Tolerance improved slightly for plant-optimized AAD-2 (v2) versus the native gene; however, comparison of both AAD-12 and AAD-2 plant optimized genes indicates a significant advantage for AAD-12 (v1) in planta.

These results are unexpected given that the in vitro comparison of AAD-2 (v1) (see PCT/US2005/014737) and AAD-12 (v2) indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. AAD-2 (v1) is expressed in individual  $T_1$  plants to varying levels; however, little protection from 2,4-D injury is afforded by this expressed protein. No substantial difference was evident in protein expression level (in planta) for the native and plant optimized AAD-2 genes (see PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of AAD-12 (v1) in planta, and resulting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides, unexpected.

#### Example 15—Preplant Burndown Applications

This and the following Examples are specific examples of novel herbicide uses made possible by the subject AAD-12 invention.

Preplant burndown herbicide applications are intended to kill weeds that have emerged over winter or early spring prior to planting a given crop. Typically these applications are applied in no-till or reduced tillage management systems where physical removal of weeds is not completed prior to planting. An herbicide program, therefore, must control a very wide spectrum of broadleaf and grass weeds present at the time of planting. Glyphosate, gramoxone, and glufosinate are examples of non-selective, non-residual herbicides widely used for preplant burndown herbicide applications. Some weeds, however, are difficult to control at this time of the season due to one or more of the following: inherent insensitivity of the weed species or biotype to the herbicide, relatively large size of winter annual weeds, and cool weather conditions limiting herbicide uptake and activity. Several herbicide options are available to tankmix with these herbicides to increase spectrum and activity on weeds where the non-selective herbicides are weak. An example would be 2,4-D tankmix applications with glyphosate to assist in the control of *Conyza canadensis* (horseweed). Glyphosate can be used from 420 to 1680 g ac/ha, more typically 560 to 840 g ae/ha, for the preplant burndown control of most weeds present; however, 280-1120 g ae/ha of 2,4-D can be applied to aid in control of many broadleaf weed species (e.g., horseweed). 2,4-D is an herbicide of choice because it is effective on a very wide range of broadleaf weeds, effective even at low temperatures, and extremely inexpensive. However, if the subsequent crop is a sensitive dicot crop, 2,4-D residues in the soil (although short-lived) can negatively impact the crop. Soybeans are a sensitive crop and require a minimum time period of 7 days (for 280 g ac/ha 2,4-D rate) to at least 30 days (for 2,4-D applications of 1120 g ae/ha) to occur between burndown applications and planting. 2,4-D is prohibited as a burndown treatment prior to cotton planting (see federal labels, most are available through CPR, 2005 or online at [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)). With AAD-12 (v1) transformed cotton or soybeans, these crops should be able to survive 2,4-D residues in the soil from burndown

86

applications applied right up to and even after planting before emergence of the crop. The increased flexibility and reduced cost of tankmix (or commercial premix) partners will improve weed control options and increase the robustness of burndown applications in important no-till and reduced tillage situations. This example is one of many options that will be available. Those skilled in the art of weed control will note a variety of other applications including, but not limited to gramoxone+2,4-D or glufosinate+2,4-D by utilizing products described in federal herbicide labels (CPR, 2005) and uses described in Agrilience Crop Protection Guide (2005), as examples. Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other phenoxy auxin herbicide) crop that would be protected by the AAD-12 (v1) gene if stably transformed. Likewise, the unique attributes of AAD-12 allowing degradation of triclopyr and fluroxypyr increase utility by allowing substitution or tank mixes of 70-1120 or 35-560 g ae/ha of triclopyr and fluroxypyr, respectively, to increase spectrum and/or increase the ability to control perennial or viney weed species.

#### Example 16—In-Crop Use of Phenoxy Auxins Herbicides in Soybeans, Cotton, and Other Dicot Crops Transformed Only with AAD-12 (v1)

AAD-12 (v1) can enable the use of phenoxy auxin herbicides (e.g., 2,4-D and MCPA) and pyridyloxy auxins (triclopyr and fluroxypyr) for the control of a wide spectrum of broadleaf weeds directly in crops normally sensitive to 2,4-D. Application of 2,4-D at 280 to 2240 g ae/ha would control most broadleaf weed species present in agronomic environments. More typically, 560-1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 g ae/ha.

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, and fluroxypyr when used at higher rates, whereas a non-residual herbicide like glyphosate would provide no control of later germinating weeds. This tool also provides a mechanism to combine herbicide modes of action with the convenience of HTC as an integrated herbicide resistance and weed shift management strategy.

A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include herbicides of the chloracetamide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

Further benefits could include tolerance to 2,4-D, triclopyr or fluroxypyr required before planting following aryloxyacetic acid auxin herbicide application (see previous example); and fewer problems from contamination injury to dicot crops resulting from incompletely cleaned bulk tanks

US 11,371,055 B2

87

that had contained 2,4-D, triclopyr or fluroxypyr. Dicamba (and many other herbicides) can still be used for the subsequent control of AAD-12 (v1)-transformed dicot crop volunteers.

Those skilled in the art will also recognize that the above example can be applied to any 2,4-D-sensitive (or other aryloxy auxin herbicide) crop that would be protected by the AAD-12 (v1) gene if stably transformed. One skilled in the art of weed control will now recognize that use of various commercial phenoxy or pyridyloxy auxin herbicides alone or in combination with a herbicide is enabled by AAD-12 (v1) transformation. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation or any commercial or academic crop protection references such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 17—In-Crop Use of Phenoxy Auxin and Pyridyloxy Auxin Herbicides in AAD-12 (v1) Only Transformed Corn, Rice, and Other Monocot Species

In an analogous fashion, transformation of grass species (such as, but not limited to, corn, rice, wheat, barley, or turf and pasture grasses) with AAD-12 (v1) would allow the use of highly efficacious phenoxy and pyridyloxy auxins in crops where normally selectivity is not certain. Most grass species have a natural tolerance to auxinic herbicides such as the phenoxy auxins (i.e., 2,4-D,.). However, a relatively low level of crop selectivity has resulted in diminished utility in these crops due to a shortened window of application timing or unacceptable injury risk. AAD-12 (v1)-transformed monocot crops would, therefore, enable the use of a similar combination of treatments described for dicot crops such as the application of 2,4-D at 280 to 2240 g ae/ha to control most broadleaf weed species. More typically, 560-1120 g ae/ha is used. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ac/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

An advantage to this additional tool is the extremely low cost of the broadleaf herbicide component and potential short-lived residual weed control provided by higher rates of 2,4-D, triclopyr, or fluroxypyr. In contrast, a non-residual herbicide like glyphosate would provide no control of later-germinating weeds. This tool would also provide a mechanism to rotate herbicide modes of action with the convenience of HTC as an integrated-herbicide-resistance and weed-shift-management strategy in a glyphosate tolerant crop/AAD-12 (v1) HTC combination strategy, whether one rotates crops species or not.

A further advantage this tool provides is the ability to tankmix broad spectrum broadleaf weed control herbicides (e.g., 2,4-D, triclopyr and fluroxypyr) with commonly used residual weed control herbicides. These herbicides are typically applied prior to or at planting, but often are less effective on emerged, established weeds that may exist in the field prior to planting. By extending the utility of these aryloxy auxin herbicides to include at-plant, preemergence, or pre-plant applications, the flexibility of residual weed control programs increases. One skilled in the art would recognize the residual herbicide program will differ based on the crop of interest, but typical programs would include

88

herbicides of the chloracetamide and dinitroaniline herbicide families, but also including herbicides such as clomazone, sulfentrazone, and a variety of ALS-inhibiting PPO-inhibiting, and HPPD-inhibiting herbicides.

The increased tolerance of corn, rice, and other monocots to the phenoxy or pyridyloxy auxins shall enable use of these herbicides in-crop without growth stage restrictions or the potential for crop leaning, unfurling phenomena such as "rat-tailing," crop leaning, growth regulator-induced stalk brittleness in corn, or deformed brace roots. Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

Example 18—AAD-12 (v1) Stacked with Glyphosate Tolerance Trait in any Crop

The vast majority of cotton, canola, corn, and soybean acres planted in North America contain a glyphosate tolerance (GT) trait, and adoption of GT corn is on the rise. Additional GT crops (e.g., wheat, rice, sugar beet, and turf) have been under development but have not been commercially released to date. Many other glyphosate resistant species are in experimental to development stage (e.g., alfalfa, sugar cane, sunflower, beets, peas, carrot, cucumber, lettuce, onion, strawberry, tomato, and tobacco; forestry species like poplar and sweetgum; and horticultural species like marigold, *petunia*, and begonias; [isb.vt.edu/cfdocs/fieldtests1.cfm](http://isb.vt.edu/cfdocs/fieldtests1.cfm), 2005 on the World Wide Web). GTC's are valuable tools for the sheer breadth of weeds controlled and convenience and cost effectiveness provided by this system. However, glyphosate's utility as a now-standard base treatment is selecting for glyphosate resistant weeds. Furthermore, weeds that glyphosate is inherently less efficacious on are shifting to the predominant species in fields where glyphosate-only chemical programs are being practiced. By stacking AAD-12 (v1) with a GT trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-12 (v1), monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and phenoxy auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and a GT trait are stacked in any monocot or dicot crop species:

- a) Glyphosate can be applied at a standard postemergent application rate (420 to 2160 g ae/ha, preferably 560 to 840 g ae/ha) for the control of most grass and broadleaf weed species. For the control of glyphosate resistant broadleaf weeds like *Conyza canadensis* or weeds inherently difficult to control with glyphosate (e.g., *Commelina* spp, *Ipomoea* spp, etc), 280-2240 g ae/ha (preferably 560-1120 g ae/ha) 2,4-D can be applied sequentially, tank mixed, or as a premix with glyphosate to provide effective control. For triclopyr, application rates would typically range from 70-1120 g ac/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.
- b) Currently, glyphosate rates applied in GTC's generally range from 560 to 2240 g ae/ha per application timing. Glyphosate is far more efficacious on grass species than broadleaf weed species. AAD-12 (v1)+GT stacked traits would allow grass-effective rates of glyphosate (105-840 g ae/ha, more preferably 210-420 g ae/ha).

US 11,371,055 B2

89

2,4-D (at 280-2240 g ae/ha, more preferably 560-1120 g ae/ha) could then be applied sequentially, tank mixed, or as a premix with grass-effective rates of glyphosate to provide necessary broadleaf weed control. Triclopyr and fluroxypyr at rates mentioned above would be acceptable components in the treatment regimen. The low rate of glyphosate would also provide some benefit to the broadleaf weed control; however, primary control would be from the 2,4-D, triclopyr, or fluroxypyr.

One skilled in the art of weed control will recognize that use of one or more commercial aryloxy auxin herbicides alone or in combination (sequentially or independently) is enabled by AAD-12 (v1) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

#### Example 19—AAD-12 (v1) Stacked with Glufosinate Tolerance Trait in any Crop

Glufosinate tolerance (PAT or bar) is currently present in a number of crops planted in North America either as a selectable marker for an input trait like insect resistance proteins or specifically as an HTC trait. Crops include, but are not limited to, glufosinate tolerant canola, corn, and cotton. Additional glufosinate tolerant crops (e.g., rice, sugar beet, soybeans, and turf) have been under development but have not been commercially released to date. Glufosinate, like glyphosate, is a relatively non-selective, broad spectrum grass and broadleaf herbicide. Glufosinate's mode of action differs from glyphosate. It is faster acting, resulting in desiccation and "burning" of treated leaves 24-48 hours after herbicide application. This is advantageous for the appearance of rapid weed control. However, this also limits translocation of glufosinate to meristematic regions of target plants resulting in poorer weed control as evidenced by relative weed control performance ratings of the two compounds in many species (Agrilience, 2005).

By stacking AAD-12 (v1) with a glufosinate tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and a glufosinate tolerance trait are stacked in any monocot or dicot crop species:

a) Glufosinate can be applied at a standard postemergent application rate (200 to 1700 g ae/ha, preferably 350 to 500 g ae/ha) for the control of many grass and broadleaf weed species. To date, no glufosinate-resistant weeds have been confirmed; however, glufosinate has a greater number of weeds that are inherently more tolerant than does glyphosate.

i) Inherently tolerant broadleaf weed species (e.g., *Cirsium arvensis*, *Apocynum cannabinum*, and *Conyza canadensis*) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-2240 g ae/ha, 2,4-D for effective control of these more difficult-to-control perennial species and to improve the robustness of control on annual broadleaf weed species. Triclopyr and fluroxypyr would be accept-

90

able components to consider in the weed control regimen. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 ae/ha.

b) A multiple combination of glufosinate (200-500 g ae/ha)+/-2,4-D (280-1120 g ae/ha)+/-triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of one or more commercial aryloxyacetic auxin herbicides alone or in combination (sequentially or independently) is enabled by AAD-12 (v1) transformation into crops. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

#### Example 20—AAD-12 (v1) Stacked with AHAS Trait in any Crop

Imidazolinone herbicide tolerance (AHAS, et al.) is currently present in a number of crops planted in North America including, but not limited to, corn, rice, and wheat. Additional imidazolinone tolerant crops (e.g., cotton and sugar beet) have been under development but have not been commercially released to date. Many imidazolinone herbicides (e.g., imazamox, imazethapyr, imazaquin, and imazapic) are currently used selectively in various conventional crops. The use of imazethapyr, imazamox, and the non-selective imazapic has been enabled through imidazolinone tolerance traits like AHAS et al. This chemistry class also has significant soil residual activity, thus being able to provide weed control extended beyond the application timing, unlike glyphosate or glufosinate-based systems. However, the spectrum of weeds controlled by imidazolinone herbicides is not as broad as glyphosate (Agrilience, 2005). Additionally, imidazolinone herbicides have a mode of action (inhibition of acetolactate synthase, ALS) to which many weeds have developed resistance (Heap, 2005). By stacking AAD-12 (v1) with an imidazolinone tolerance trait, either through conventional breeding or jointly as a novel transformation event, weed control efficacy, flexibility, and ability to manage weed shifts and herbicide resistance development could be improved. As mentioned in previous examples, by transforming crops with AAD-12 (v1), monocot crops will have a higher margin of phenoxy or pyridyloxy auxin safety, and these auxins can be selectively applied in dicot crops. Several scenarios for improved weed control options can be envisioned where AAD-12 (v1) and an imidazolinone tolerance trait are stacked in any monocot or dicot crop species:

a) Imazethapyr can be applied at a standard postemergent application rate of (35 to 280 g ae/ha, preferably 70-140 g ae/ha) for the control of many grass and broadleaf weed species.



## US 11,371,055 B2

91

- i) ALS-inhibitor resistant broadleaf weeds like *Amaranthus rudis*, *Ambrosia trifida*, *Chenopodium album* (among others, Heap, 2005) could be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. For triclopyr, application rates would typically range from 70-1120 g ae/ha, more typically 140-420 g ae/ha. For fluroxypyr, application rates would typically range from 35-560 g ae/ha, more typically 70-280 g ae/ha.
- ii) Inherently more tolerant broadleaf species to imidazolinone herbicides like *Ipomoea* spp. can also be controlled by tank mixing 280-2240 g ae/ha, more preferably 560-1120 g ae/ha, 2,4-D. See rates above for triclopyr or fluroxypyr.
- b) A multiple combination of imazethapyr (35 to 280 g ae/ha, preferably 70-140 g ae/ha)+/-2,4-D (280-1120 g ae/ha)+/-triclopyr or fluroxypyr (at rates listed above), for example, could provide more robust, overlapping weed control spectrum. Additionally, the overlapping spectrum provides an additional mechanism for the management or delay of herbicide resistant weeds.

One skilled in the art of weed control will recognize that use of any of various commercial imidazolinone herbicides, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by AAD-12 (v1) transformation and stacking with any imidazolinone tolerance trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTC's by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

## Example 21—AA4D-12 (v1) in Rice

## 21.1—Media Description.

Culture media employed were adjusted to pH 5.8 with 1 M KOH and solidified with 2.5 g/L Phytigel (Sigma). Embryogenic calli were cultured in 100x20 mm Petri dishes containing 40 ml semi-solid medium. Rice plantlets were grown on 50 ml medium in Magenta boxes. Cell suspensions were maintained in 125-ml conical flasks containing 35 ml liquid medium and rotated at 125 rpm. Induction and maintenance of embryogenic cultures took place in the dark at 25-26° C., and plant regeneration and whole-plant culture took place in a 16-h photoperiod (Zhang et al. 1996).

Induction and maintenance of embryogenic callus took place on NB basal medium as described previously (Li et al. 1993), but adapted to contain 500 mg/L glutamine. Suspension cultures were initiated and maintained in SZ liquid medium (Zhang et al. 1998) with the inclusion of 30 g/L sucrose in place of maltose. Osmotic medium (NBO) consisted of NB medium with the addition of 0.256 M each of mannitol and sorbitol. Hygromycin-B-resistant callus was selected on NB medium supplemented with 50 mg/L hygromycin B for 3-4 weeks. Pre-regeneration took place on medium (PRH50) consisting of NB medium without 2,4-dichlorophenoxyacetic acid (2,4-D), but with the addition of 2 mg/L 6-benzylaminopurine (BAP), 1 mg/L  $\alpha$ -naphthaleneacetic acid (NAA), 5 mg/L abscisic acid (ABA) and 50 mg/L hygromycin B for 1 week. Regeneration of plantlets followed via culture on regeneration medium (RNH50)

92

comprising NB medium without 2,4-D, and supplemented with 3 mg/L BAP, 0.5 mg/L NAA, and 50 mg/L hygromycin B until shoots regenerated. Shoots were transferred to rooting medium with half-strength Murashige and Skoog basal salts and Gamborg's B5 vitamins, supplemented with 1% sucrose and 50 mg/L hygromycin B (½MSH50).

## 21.2—Tissue Culture Development.

Mature desiccated seeds of *Oryza sativa* L. *japonica* cv. Taipei 309 were sterilized as described in Zhang et al. 1996. Embryogenic tissues were induced by culturing sterile mature rice seeds on NB medium in the dark. The primary callus approximately 1 mm in diameter, was removed from the scutellum and used to initiate cell suspension in SZ liquid medium. Suspensions were then maintained as described in Zhang 1995. Suspension-derived embryogenic tissues were removed from liquid culture 3-5 days after the previous subculture and placed on NBO osmotic medium to form a circle about 2.5 cm across in a Petri dish and cultured for 4 h prior to bombardment. Sixteen to 20 h after bombardment, tissues were transferred from NBO medium onto NBH50 hygromycin B selection medium, ensuring that the bombarded surface was facing upward, and incubated in the dark for 14-17 days. Newly formed callus was then separated from the original bombarded explants and placed nearby on the same medium. Following an additional 8-12 days, relatively compact, opaque callus was visually identified, and transferred to PRH50 pre-regeneration medium for 7 days in the dark. Growing callus, which became more compact and opaque was then subcultured onto RNH50 regeneration medium for a period of 14-21 days under a 16-h photoperiod. Regenerating shoots were transferred to Magenta boxes containing ½ MSH50 medium. Multiple plants regenerated from a single explant are considered siblings and were treated as one independent plant line. A plant was scored as positive for the hph gene if it produced thick, white roots and grew vigorously on ½ MSH50 medium. Once plantlets had reached the top of Magenta boxes, they were transferred to soil in a 6-cm pot under 100% humidity for a week, then moved to a growth chamber with a 14-h light period at 30° C. and in the dark at 21° C. for 2-3 weeks before transplanting into 13-cm pots in the greenhouse. Seeds were collected and dried at 37° C. for one week, prior to storage.

## 21.3—Microprojectile Bombardment.

All bombardments were conducted with the Biolistic PDS-1000/He™ system (Bio-Rad, Laboratories, Inc.). Three milligrams of 1.0 micron diameter gold particles were washed once with 100% ethanol, twice with sterile distilled water and resuspended in 50  $\mu$ l water in a siliconized Eppendorf tube. Five micrograms plasmid DNA representing a 1:6 molar ratio of pDOW3303(Hpt-containing vector) to pDAB4101 (AAD-12 (v1)+AHAS). 20  $\mu$ l spermidine (0.1 M) and 50  $\mu$ l calcium chloride (2.5 M) were added to the gold suspension. The mixture was incubated at room temperature for 10 min, pelleted at 10000 rpm for 10 s, resuspended in 60  $\mu$ l cold 100% ethanol and 8-9  $\mu$ l was distributed onto each macrocarrier. Tissue samples were bombarded at 1100 psi and 27 in of Hg vacuum as described by Zhang et al. (1996).

21.4—Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T<sub>0</sub> Rice

Rice plantlets at the 3-5 leaf stage were sprayed with a lethal dose of 0.16% (v/v) solution of Pursuit (to confirm the presence of the AHAS gene) containing 1% Sunit II (v/v) and 1.25% UAN (v/v) using a track sprayer calibrated to 187 L/ha. Rating for sensitivity or resistance was performed at 36 days after treatment (DAT). Ten of the 33 events sent to

US 11,371,055 B2

93

the greenhouse were robustly tolerant to the Pursuit; others suffered varying levels of herbicide injury. Plants were sampled (according to section 21.7 below) and molecular characterization was performed as previously described in Example 8 that identified seven of these 10 events as containing both the AAD-12 (v1) PTU and the entire AHAS coding region.

#### 21.5—Heritability of AAD-12 (v1) in T<sub>1</sub> Rice

A 100-plant progeny test was conducted on five T<sub>1</sub> lines of AAD-12 (v1) lines that contained both the AAD-12 (v1) PTU and AHAS coding region. The seeds were planted with respect to the procedure above and sprayed with 140 g ae/ha imazethapyr using a track sprayer as previously described. After 14 DAT, resistant and sensitive plants were counted. Two out of the five lines tested segregated as a single locus, dominant Mendelian trait (3R:1S) as determined by Chi square analysis. AAD-12 coseregated with the AHAS selectable marker as determined by 2,4-D tolerance testing below.

#### 21.6—Verification of High 2,4-D Tolerance in T<sub>1</sub> Rice.

The following T<sub>1</sub> AAD-12 (v1) single segregating locus lines were planted into 3-inch pots containing Metro Mix media: pDAB4101(20)003 and pDAB4101(27)002. At 2-3 leaf stage were sprayed with 140 g ae/ha imazethapyr. Nulls were eliminated and individuals were sprayed at V3-V4 stage in the track sprayer set to 187 L/ha at 1120, 2240 or 4480 g ac/ha 2,4-D DMA (2×, 4×, and 8× typical commercial use rates, respectively). Plants were graded at 7 and 14 DAT and compared to untransformed commercial rice cultivar, 'Lamont,' as negative control plants.

Injury data (Table 27) shows that the AAD-12 (v1)-transformed lines are more tolerant to high rates of 2,4-D DMA than the untransformed controls. The line pDAB4101(20)003 was more tolerant to high levels of 2,4-D than the line pDAB4101(27)002. The data also demonstrates that tolerance of 2,4-D is stable for at least two generations.

TABLE 27

T <sub>1</sub> AAD-12 (v1) and untransformed control response to varying levels of 2,4-D DMA.			
Herbicide	Lemont Untransformed Control	pDAB4101(20)003 Average % Injury 14 DAT	pDAB4101(27)002
1120 g ae/ha 2,4-D DMA	20	10	10
2240 g ae/ha 2,4-D DMA	35	15	30
4480 g ae/ha 2,4-D DMA	50	23	40

#### 21.7—Tissue Harvesting, DNA Isolation and Quantification.

Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, Dneasy 69109). An aliquot of the

94

extracted DNA was then stained with Pico Green (Molecular Probes P7589) and scanned in the fluorometer (BioTek) with known standards to obtain the concentration in ng/μl.

#### 21.8—AAD-12 (v1) Expression.

Sample preparation and analysis conditions were as described previously. All 33 T<sub>0</sub> transgenic rice lines and 1 non-transgenic control were analyzed for AAD-12 expression using ELISA blot. AAD-12 was detected in the clones of 20 lines, but not in line Taipai 309 control plant. Twelve of the 20 lines that had some of the clones tolerant to imazethapyr were expressing AAD-12 protein, were AAD-12 PCR PTU positive, and AHAS coding region positive. Expression levels ranged from 2.3 to 1092.4 ppm of total soluble protein.

#### 21.9—Field Tolerance of pDAB4101 Rice Plants to 2,4-D and Triclopyr Herbicides.

A field level tolerance trial was conducted with AAD-12 (v1) event pDAB4101[20] and one wild-type rice (Clearfield 131) at Wayside, Miss. (a non-transgenic imidazolinone-resistant variety). The experimental design was a randomized complete block design with a single replication. Herbicide treatments were 2× rates of 2,4-D (dimethylamine salt) at 2240 g ae/ha and triclopyr at 560 g ae/ha plus an untreated control. Within each herbicide treatment, two rows of T<sub>1</sub> generation pDAB4101[20] and two rows of Clearfield rice were planted using a small plot drill with 8-inch row spacing. The pDAB4101[20] rice contained the AHAS gene as a selectable marker for the AAD-12(v1) gene. Imazethapyr was applied at the one leaf stage as selection agent to remove any AAD-12 (v1) null plants from the plots. Herbicide treatments were applied when the rice reached the 2 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual ratings of injury were taken at 7, 14 and 21 days after application.

AAD-12 (v1) event response to 2,4-D and triclopyr are shown in Table 28. The non-transformed rice line (Clearfield) was severely injured (30% at 7DAT and 35% at 15DAT) by 2,4-D at 2240 g ae/ha which is considered the 4× commercial use rate. The AAD-12 (v1) event demonstrated excellent tolerance to 2,4-D with no injury observed at 7 or 15DAT. The non-transformed rice was significantly injured (15% at 7DAT and 25% at 15DAT) by the 2× rate of triclopyr (560 g ae/ha). The AAD-12 (v1) event demonstrated excellence tolerance to the 2× rates of triclopyr with no injury observed at either 7 or 15DAT.

These results indicate that the AAD-12 (v1) transformed rice displayed a high level of resistance to 2,4-D and triclopyr at rates that caused severe visual injury to the Clearfield rice. It also demonstrates the ability to stack multiple herbicide tolerance genes with AAD-12 I multiple species to provide resistance to a wider spectrum of effective chemistries

US 11,371,055 B2

95

96

TABLE 28

AAD-12 T <sub>1</sub> generation rice plants response to 2,4-D and triclopyr under field conditions.					
		% Visual injury			
		7DAT		15DAT	
Herbicide Treatment		AAD-12	Wild-		
Active Ingredient	Rate	event pDAB4101[20]	type Clearfield	AAD-12 event pDAB4101[20]	Wild-type Clearfield
2,4-D	2240 GM AE/HA	0	15	0	35
Triclopyr	840 GM AE/HA	0	30	0	25
Untreated		0	0	0	0

## Example 22—AAD-12 (v1) in Canola

## 22.1—Canola Transformation.

The AAD-12 (v1) gene conferring resistance to 2,4-D was used to transform *Brassica napus* var. Nexera\* 710 with *Agrobacterium*-mediated transformation and plasmid pDAB3759. The construct contained AAD-12 (v1) gene driven by CsVMV promoter and Pat gene driven by AtUbi10 promoter and the EPSPS glyphosate resistance trait driven by AtUbi10 promoter (see section 2.4).

Seeds were surface-sterilized with 10% commercial bleach for 10 minutes and rinsed 3 times with sterile distilled water. The seeds were then placed on one half concentration of MS basal medium (Murashige and Skoog, 1962) and maintained under growth regime set at 25° C., and a photoperiod of 16 hrs light/8 hrs dark.

Hypocotyl segments (3-5 mm) were excised from 5-7 day old seedlings and placed on callus induction medium K1D1 (MS medium with 1 mg/L kinetin and 1 mg/L 2,4-D) for 3 days as pre-treatment. The segments were then transferred into a petri plate, treated with *Agrobacterium* Z707S or LBA4404 strain containing pDAB3759. The *Agrobacterium* was grown overnight at 28° C. in the dark on a shaker at 150 rpm and subsequently re-suspended in the culture medium.

After 30 min treatment of the hypocotyl segments with *Agrobacterium*, these were placed back on the callus induction medium for 3 days. Following co-cultivation, the segments were placed on K1D1TC (callus induction medium containing 250 mg/L Carbenicillin and 300 mg/L Timentin) for one week or two weeks of recovery. Alternately, the segments were placed directly on selection medium K1D1HI (above medium with 1 mg/L Herbicide). Carbenicillin and Timentin were the antibiotics used to kill the *Agrobacterium*. The selection agent Herbicide allowed the growth of the transformed cells.

Callused hypocotyl segments were then placed on B3Z1H1 (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 1 mg/L Herbicide, Carbenicillin and Timentin) shoot regeneration medium. After 2-3 weeks shoots started regenerating. Hypocotyl segments along with the shoots are transferred to B3Z1H3 medium (MS medium, 3 mg/L benzylamino purine, 1 mg/L Zeatin, 0.5 gm/L MES [2-(N-morpholino) ethane sulfonic acid], 5 mg/L silver nitrate, 3 mg/L Herbicide, Carbenicillin and Timentin) for another 2-3 weeks.

Shoots were excised from the hypocotyl segments and transferred to shoot elongation medium MESH5 or MES10 (MS, 0.5 gm/L MES, 5 or 10 mg/L Herbicide, Carbenicillin, Timentin) for 2-4 weeks. The elongated shoots are cultured

for root induction on MSI.1 (MS with 0.1 mg/L Indolebutyric acid). Once the plants had a well established root system, these were transplanted into soil. The plants were acclimated under controlled environmental conditions in the Conviron for 1-2 weeks before transfer to the greenhouse.

## 22.2—Molecular Analysis: Canola Materials and Methods

22.2.1—Tissue Harvesting DNA Isolation and Quantification. Fresh tissue was placed into tubes and lyophilized at 4° C. for 2 days. After the tissue was fully dried, a tungsten bead (Valenite) was placed in the tube and the samples were subjected to 1 minute of dry grinding using a Kelco bead mill. The standard DNeasy DNA isolation procedure was then followed (Qiagen, DNeasy 69109). An aliquot of the extracted DNA was then stained with Pico Green (Molecular Probes P7589) and read in the fluorometer (BioTek) with known standards to obtain the concentration in ng/ul.

22.2.2—Polymerase Chain Reaction. A total of 100 ng of total DNA was used as the template. 20 mM of each primer was used with the Takara Ex Taq PCR Polymerase kit (Mirus TAKRR001A). Primers for Coding Region PCR AAD-12 (v1) were (SEQ ID NO:10) (forward) and (SEQ ID NO:11) (reverse). The PCR reaction was carried out in the 9700 Geneamp thermocycler (Applied Biosystems), by subjecting the samples to 94° C. for 3 minutes and 35 cycles of 94° C. for 30 seconds, 65° C. for 30 seconds, and 72° C. for 2 minutes followed by 72° C. for 10 minutes. PCR products were analyzed by electrophoresis on a 1% agarose gel stained with EtBr. 35 samples from 35 plants with AAD-12 (v1) events tested positive. Three negative control samples tested negative.

## 22.2.3—ELISA.

Using established ELISA described in previous section, AAD-12 protein was detected in 5 different canola transformation plant events. Expression levels ranged from 14 to over 700 ppm of total soluble protein (TSP). Three different untransformed plant samples were tested in parallel with no signal detected, indicating that the antibodies used in the assay have minimal cross reactivity to the canola cell matrix. These samples were also confirmed positive by Western analysis. A summary of the results is presented in Table 29.

TABLE 29

Expression of AAD-12 (v1) in Canola plants				
Sample #	[TSP] (µg/ml)	[AAD-12] (ng/ml)	Expression (ppm TSP) (ELISA)	Western
31	5614.96	1692.12	301.36	++++
33	4988.26	2121.52	425.30	++++
38	5372.25	3879.09	722.06	++++
39	2812.77	41.36	14.71	+

US 11,371,055 B2

97

TABLE 29-continued

Expression of AAD-12 (v1) in Canola plants				
Sample #	[TSP] (µg/ml)	[AAD-12] (ng/ml)	Expression (ppm TSP) (ELISA)	Western
40	3691.48	468.74	126.98	+++
Control 1	2736.24	0.00	0.00	-
Control 2	2176.06	0.00	0.00	-
Control 3	3403.26	0.00	0.00	-

22.4—Postemergence Herbicide Tolerance in AAD-12 (v1) Transformed T<sub>0</sub> Canola.

Forty-five T<sub>0</sub> events from the transformed with the construct pDAB3759, were sent to the greenhouse over a period of time and were allowed to acclimate in the greenhouse. The plants were grown until 2-4 new, normal looking leaves

98

Surviving plants were transplanted into 3-inch pots containing Metro Mix media. Surviving plants from T<sub>1</sub> progenies, that were selected with 560 g ae/ha 2,4-D, were also transplanted into 3-inch pots filled with Metro Mix soil. At 2-4 leaf stage plants were sprayed with either 280, 560, 1120, or 2240 g ae/ha 2,4-D DMA. Plants were graded at 3 and 14 DAT and compared to untransformed control plants. A sampling of T<sub>1</sub> event injury data 14DAT may be seen in Table 30. Data suggests that multiple events are robustly resistant to 2240 g ae/ha 2,4-D, while other events demonstrated less robust tolerance up to 1120 g ae/ha 2,4-D. Surviving plants were transplanted to 5¼" pots containing Metro Mix media and placed in the same growth conditions as before and self-pollinated to produce only homozygous seed.

TABLE 30

T <sub>1</sub> AAD-12 (v1) and untransformed control response to varying rates postemergence 2,4-D DMA applications.						
Herbicide	Untransformed Control	pDAB3759(33) 013.001	pDAB3759(18) 009.001	pDAB3759(18) 022.001	pDAB3759(18) 030.001	pDAB3759(18) 023.001
Average % Injury 14DAT						
280 g ae/ha 2,4-D DMA	85	0	0	0	0	0
560 g ae/ha 2,4-D DMA	85	0	0	0	0	0
1120 g ae/ha 2,4-D DMA	90	0	0	13	5	3
2240 g ae/ha 2,4-D DMA	95	1	5	83	31	6

had emerged (i.e., plants had transitioned from tissue culture to greenhouse growing conditions). Plants were then treated with a lethal dose of the commercial formulations of 2,4-D Amine 4 at a rate of 560 g ae/ha. Herbicide applications were made with a track sprayer at a spray volume of 187 L/ha, 50-cm spray height. A lethal dose is defined as the rate that causes >95% injury to the untransformed controls.

Twenty-four of the events were tolerant to the 2,4-D DMA herbicide application. Some events did incur minor injury but recovered by 14 DAT. Events were progressed to the T<sub>1</sub> (and T<sub>2</sub> generation) by self pollination under controlled, bagged, conditions.

22.5—AAD-12 (v1) Heritability in Canola.

A 100 plant progeny test was also conducted on 11 T<sub>1</sub> lines of AAD-12 (v1). The seeds were sown and transplanted to 3-inch pots filled with Metro Mix media. All plants were then sprayed with 560 g ae/ha 2,4-D DMA as previously described. After 14 DAT, resistant and sensitive plants were counted. Seven out of the 11 lines tested segregated as a single locus, dominant Mendelian trait (3R: S) as determined by Chi-square analysis. AAD-12 is heritable as a robust aryloxyalkanoate auxin resistance gene in multiple species and can be stacked with one or more additional herbicide resistance genes.

22.6—Verification of High 2,4-D Tolerance in T<sub>1</sub> Canola

For T<sub>1</sub> AAD-12 (v1), 5-6 mg of seed were stratified, sown, and a fine layer of Sunshine Mix #5 media was added as a top layer of soil. Emerging plants were selected with 560 g ae/ha 2,4-D at 7 and 13 days after planting.

22.7—Field tolerance of pDAB3759 canola plants to 2,4-D, dichloprop, triclopyr and fluroxypyr herbicides.

Field level tolerance trial was conducted on two AAD-12 (v1) events 3759(20)018.001 and 3759(18)030.001 and a wild-type canola (Nex710) in Fowler, Ind. The experimental design was a randomized complete block design with 3 replications. Herbicide treatments were 2,4-D (dimethylamine salt) at 280, 560, 1120, 2240 and 4480 g ac/ha, triclopyr at 840 g ae/ha, fluroxypyr at 280 g ae/ha and an untreated control. Within each herbicide treatment, single 20 ft row/event for event 3759(18)030.0011, 3759(18)018.001 and wild-type line (Nex710) were planted with a 4 row drill on 8 inch row spacing. Herbicide treatments were applied when canola reached the 4-6 leaf stage using compressed air backpack sprayer delivering 187 L/ha carrier volume at 130-200 kpa pressure. Visual injury ratings were taken at 7, 14 and 21 days after application.

Canola response to 2,4-D, triclopyr, and fluroxypyr are shown in Table 31. The wild-type canola (Nex710) was severely injured (72% at 14DAT) by 2,4-D at 2240 g ac/ha which is considered the 4x rate. The AAD-12 (v1) events all demonstrated excellent tolerance to 2,4-D at 14DAT with an average injury of 2, 3 and 2% observed at the 1, 2 and 4x rates, respectively. The wild-type canola was severely injured (25% at 14DAT) by the 2x rate of triclopyr (840 g ac/ha). AAD-12 (v1) events demonstrated tolerance at 2x rates of triclopyr with an average of 6% injury at 14DAT across the two events. Fluroxypyr at 280 g ae/ha caused severe injury (37%) to the non-transformed line at 14DAA. AAD-12 (v1) events demonstrated increased tolerance with an average of 8% injury at 5DAT.

These results indicate that AAD-12 (v1) transformed events displayed a high level of resistance to 2,4-D, triclopyr and fluroxypyr at rates that were lethal or caused severe epinastic malformations to non-transformed canola. AAD-12 has been shown to have relative efficacy of 2,4-D>triclopyr>fluroxypyr.



US 11,371,055 B2

99

TABLE 31

AAD-12 (pDAB3759) canola plants response to 2,4-D, triclopyr, and fluroxypyr under field conditions.				
Herbicide Treatment		% Visual Injury at 14 DAT		
Active Ingredient	Rate	AAD-12 event 3759(20)018.001	AAD-12 event 3759(18)030.001	Wild Type (Nex710)
2,4-D	280 GM AE/HA	0 a	0 b	0 e
2,4-D	560 GM AE/HA	0 a	0 b	15 d
2,4-D	1120 GM AE/HA	2 a	2 ab	33 bc
2,4-D	2240 GM AE/HA	3 a	3 ab	48 a
Triclopyr	840 GM AE/HA	6 a	6 ab	25 cd
Fluroxypyr	280 GM AE/HA	7 a	8 a	37 ab

Means with a column with different letters are significantly different as defined by LSD (p = 0.05).

#### Example 23—AAD-12 (v1) Stacked with Insect Resistance (IR) or Other Input Traits in any Crop

Insect resistance in crops supplied by a transgenic trait is prevalent in corn and cotton production in North America and across the globe. Commercial products having combined IR and HT traits have been developed by multiple seed companies. These include Bt IR traits (e.g. Bt toxins listed at the website [lifesci.sussex.ac.uk](http://lifesci.sussex.ac.uk), 2006) and any or all of the HTC traits mentioned above. The value this offering brings is the ability to control multiple pest problems through genetic means in a single offering. The convenience of this offering will be restricted if weed control and insect control are accomplished independent of each other. AAD-12 (v1) alone or stacked with one or more additional HTC traits can be stacked with one or more additional input traits (e.g., insect resistance, fungal resistance, or stress tolerance, et al.) ([isb.vt.edu/cfdocs/fieldtests1.cfm](http://isb.vt.edu/cfdocs/fieldtests1.cfm), 2005) either through conventional breeding or jointly as a novel transformation event. Benefits include the convenience and flexibility described in Examples 15-20 above, together with the ability to manage insect pests and/or other agronomic stresses in addition to the improved weed control offered by AAD-12 and associated herbicide tolerance. Thus, the subject invention can be used to provide a complete agronomic package of improved crop quality with the ability to flexibly and cost effectively control any number of agronomic issues.

Combined traits of IR and HT have application in most agronomic and horticultural/ornamental crops and forestry. The combination of AAD-12 and its commensurate herbicide tolerance and insect resistance afforded by any of the number of Bt or non-Bt IR genes are can be applied to the crop species listed (but not limited to) in Example 13. One skilled in the art of weed control will recognize that use of any of various commercial herbicides described in Examples 18-20, phenoxyacetic or pyridyloxyacetic auxin herbicides, alone or in multiple combinations, is enabled by AAD-12 (v1) transformation and stacking with the corresponding HT trait or IR trait either by conventional breeding or genetic engineering. Specific rates of other herbicides representative of these chemistries can be determined by the herbicide labels compiled in the CPR (Crop Protection Reference) book or similar compilation, labels compiled online (e.g., [cdms.net/manuf/manuf.asp](http://cdms.net/manuf/manuf.asp)), or any commercial or academic crop protection guides such as the Crop Protection

100

Guide from Agrilience (2005). Each alternative herbicide enabled for use in HTCs by AAD-12 (v1), whether used alone, tank mixed, or sequentially, is considered within the scope of this invention.

#### Example 24—AAD-12 (v1) as an In Vitro Dicot Selectable Marker

Genetic engineering of plant cell, tissue, organ, and plant or organelle such as plastid starts with the process of inserting genes of interest into plant cells using a suitable delivery method. However, when a gene is delivered to plant cells, only an extremely small percentage of cells integrate the heterogeneous gene into their genome. In order to select those few cells that have incorporated the gene of interest, researchers link a selectable or screenable “marker gene” to the gene of interest (GOT) in the vector. Cells that contain these markers are identified from the whole population of cells/tissue to which the DNA plasmid vector was delivered. By selecting those cells that express the marker gene, researchers are able to identify those few cells that may have incorporated the GOI into their genome.

There are a variety of selectable markers available to enable this selection process to obtain transgenic cells, callus, embryos, shoots and plantlets. The preferred selectable markers by the Ag-industry are herbicide markers that allow the ease of spraying compounds in the field to select the right transgenic progenies during the process of event sorting in the field situation. AAD-12 (v1) has been shown to efficiently serve as a selectable marker for whole plants transformed with the gene in the greenhouse and growth chamber (Example 7) with 2,4-D as the selection agent. Field selection is possible as well using 2,4-D in combination with the AAD-12 (v1) gene (Example 11, 22), but use in vitro for cell-level selection is complicated by the fact 2,4-D is used almost ubiquitously as a plant growth regulator in the plant tissue culture systems. Degradation of this important hormone by AAD-12 (v1) can impact the ability to use this gene as an in vitro selectable marker. Success of developing 2,4-D as a marker gene depends on identifying the right alternate plant growth regulator that can mimic the effect of 2,4-D in the respective culture system and at the same time possess the ability to be stable and not be degraded by the AAD-12 enzyme when expressed in the transgenic cells. R-dichlorprop is a close analog to 2,4-D that is not a substrate for AAD-12 (v1) and is used a non-metabolizable auxin substitute in tobacco cell cultures allowing 2,4-D to be used at high rates as a selection agent. This fact was used in exemplifying AAD-12 (v1) could be used as a selectable marker in vitro.

##### 24.1—Cell Culture—Alternative Auxins.

AAD-12 (v1) degrades 2,4-D, but not R-2,4-dichlorophenoxypropionic acid (R-dichlorprop), which has at the same time the structural requirement of an auxinic growth regulator. Other non-metabolizable plant auxin mimics that may be used in cell culture include NAA (naphthalene acetic acid), IAA (indole acetic acid), dicamba, picloram, and R-mecoprop. It was investigated if it was possible to substitute R-dichlorprop and successfully maintain two different tobacco cell cultures PHL (Petite Havana) and BY2 suspensions. Conversely, for cotton explants R-dichlorprop, dicamba, and picloram were tested as alternative auxins and the embryogenic callus induction response in comparison to the standard growth regulator, 2,4-D was evaluated. Petite Havana tobacco (PHL) and Coker cotton cotyledons were used in their experiments.

US 11,371,055 B2

## 101

24.1.1—Tobacco Cell Suspension—2,4-D as Selection Agent.

A dose response study was conducted with both the R-dichlorprop habituated PHL cells and the R-dichlorprop habituated BY2 cells where R-dichlorprop was substituted directly for 2,4-D in culture media. Though the focus was on PHL, a dose response was also done with BY2 in case of possible future studies, as well as to help predict the dose response for PHL. For the dichlorprop habituated PHL dose response, the levels of 2,4-D used (on LSHY2C medium with R-dichlorprop) were 0 (the control), 1, 2, 3, 5, 8, 10, 12, 15, 18, 20, 40, 60, 80, 100, 110, 120 mg/L 2,4-D. There were four replications per concentration. For the R-dichlorprop habituated BY2 dose response, the levels of 2,4-D used (on LSHY2C medium) were 0 (the control), 1, 2, 3, 5, 8, 10, 20, 30, 40, mg/L 2,4-D.

The dose response was carried out showed that all the concentration of 2,4-D tested were lethal above 10 mg/L concentrations. However, there was growth in all the concentrations up to 10-mg/L 2,4-D where a slight growth of PHL suspension was observed. The growth of the suspension colonies from 1-8 mg/L 2,4-D concentrations was comparable to the growth in control treatments. The observation made in BY2 suspension cells were similar except the concentration at 10 mg/L was found to be lethal and the sub-lethal concentration was 8 mg/L concentration.

24.1.2—Tobacco Cell Transformation with AAD-12 (v1) and 2,4-D Selection.

For tobacco transformation experiment, there were 11 treatments altogether: a control set plated on LS-BY2C+ dichlorprop medium, and 10 sets of LSHY2C+dichlorprop+ 2,4-D at varying concentration levels (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L). There were four replications per treatment. The plasmid DNA vector used was pDAB724, and the vector used for transformation was EHA101S strain of *Agrobacterium tumefaciens*. Four ml of PHL suspension at 0.6 OD<sub>660</sub> were mixed with 100 ul of *Agrobacterium* (either EHA101 or LBA4404 strains) suspension at 1.0 OD<sub>660</sub> in a sterile Petri plate and were mixed thoroughly and co-cultivated together in a non-shake condition at a dark growth chamber for 3 days at 25° C. After the co-cultivation period 1.5 ml of the Agro-tobacco suspension mixture was plated to the 11 set of plates above. The experiment was repeated with 13 treatments: a control of LS-BY2C+dichlorprop media (no 2,4-D), and LS-BY2C+dichlorprop+2,4-D (1, 2, 3, 5, 8, 10, 12, 15, 18, 20 mg/L); LSHY2C+1 mg/L 2,4-D+B10 (Bialophos); LSHY2C+10 2,4-D+B10+R-dichlorprop. Again, there were four replicates per treatment, as well as a positive and negative control. All media contain 500 mg/L Carbenicillin (C) to control to contain *Agrobacterium* growth in the selection media.

The plasmid used in these experiments is pDAB724 and it has PAT selectable marker as well. So, control transformation experiments were initiated using R-dichlorprop habituated PHL in the presence of 10 mg/L bialophos following the standard protocol described above. The treatments were done side by side with 4 replicates to see if the bialophos selection in these suspension is normal.

There was little growth observed in all selection concentrations of 2,4D tested above 10 mg/L; however several fast growing colonies were found in 2, 5, and 8 mg/L 2,4-D concentration and representative sample was transferred to fresh selection at 10 mg/L selection to bulk the callus. Also, several putative colonies were selected in from 12, 15, 18 and 20 mg/L 2,4-D, but when compared to 10 mg/L there were only few colonies in these selection plate. Control treatment conducted with bialophos selection showed nor-

## 102

mal colony development. It appears that 10 mg/L 2,4-D is the sub-lethal and above this concentration 2,4-D appears to be lethal to the non-transformed cells. All the identified colonies were transferred to fresh medium with 10 mg/L selection and were probed for the presence of transgene by PCR as described in Example 10. The colonies selected and bulked had the transgenes as determined by PCR and expression of the genes as established by the Western analyses (as described in example 10). Several colonies were identified as actively growing and transferred to fresh selection medium with 10 mg/L 2,4-D to bulk the callus.

The bulked calluses were then transferred to higher level of 2,4-D to test the tolerance level in vitro. The levels of 2,4-D used were 20, 40, 60, 80, 100, and 120 mg/L 2,4-D. However the callus did not grow beyond 20 mg/L 2,4-D concentrations indicating a threshold concentration higher than 20 mg/L may exist.

24.2.1—Cotton Explants—Auxin Alternatives

A dose response study was initiated to test multiple auxin alternatives as a substitute for the use of 2,4-D as a growth regulator in cotton. The alternative auxin tested were 2,4-dichlorprop, dicamba, and picloram. These compounds were tested at 0.2, 2.0, and 20.0 uM concentrations respectively. 2,4-D was used as the control treatment at 0.02 uM concentration. The medium used is the base medium for cotton callus induction (Example 12). Beyond the initial phase of culture, auxin is removed from the medium to prod the tissue toward the regeneration process.

R-dichlorprop was not effective in callus induction of cotyledonary segments and appears toxic to cotton cells at the lowest concentration tested (0.02 uM). Dicamba effectively induces callus growth at all concentrations tested (0.02-20 uM) and has no apparent toxic effects in this concentration range. Callus induction with picloram increased up to a maximum when explants were treated with 0.2 uM to 20 uM. Quality of the callus was consistent with the standard 2,4-D treatment at the 2 uM picloram concentration. At the highest concentration (20 uM) 2,4-D was also inhibitory to cotton callus generation and growth.

Cotton has shown initial ability to respond effectively to alternative auxins (to 2,4-D) in culture. At high enough concentrations, 2,4-D is toxic to cotton cotyledonary explants. R-dichlorprop is surprisingly significantly more toxic to cotton than 2,4-D or other auxins. 2,4-D may be used as a selection agent and in combination with AAD-12 (v1) as the selectable marker gene. Other non-metabolizable auxin surrogates (e.g., dicamba, picloram, R-mecoprop, NAA, or IAA) would allow the use of AAD-12 as a selectable marker in dicots with 2,4-D as the selection agent.

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103

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US 11,371,055 B2

107

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108

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## SEQUENCE LISTING

&lt;160&gt; NUMBER OF SEQ ID NOS: 21

&lt;210&gt; SEQ ID NO 1

&lt;211&gt; LENGTH: 879

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Delftia acidovorans

&lt;400&gt; SEQUENCE: 1

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gtgcacctgg ccacgctgga cgacgccggc ttccgccccc tgcacgccgc ctgggtgcag    120
catgcgctgc tgatcttccc cggccagcac ctccagcaac accagcagat cacttttgcc    180
aaacgcttcg gcgcgatcga gcgcgatcgc ggccggcgaca tcgtggccat ctccaatgtc    240
aaggccgatg gcacggtgag ccagcacagc ccgcgcgagt gggacgacat gatgaaggtc    300
atcgctcgga acatggcctg gcacgcccgc agcacctaca tgccggtgat ggccgagggc    360
gcgggtgtct cggccgaagt ggtgcccgcg gtggcggggc gcacctgctt cgccgacatg    420
cgccgcgcct acgacgcgct ggacgaggcc acccgcgccc tgggtgacca gcgctcgggc    480
cggcattcgc tgggtgtatt gcagagcaag ctggggccatg tgcagcaggc cggctcgggc    540
```

US 11,371,055 B2

109

110

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```
tacatcggt acggcatgga caccaccgcc acgcccctgc gcccgtggt caaggtgcat 600
cccgagaccg gccgcccctc gctgctgata gcccgccatg cccatgccat cccgggcatg 660
gacgcccgcg aatccgagcg ctctctggaa ggctgggtcg actgggctg ccaggcgccg 720
cgggtgcatg cccaccaatg ggccgcccgc gacgtggtgg tgtgggacaa ccgtgctg 780
ctgcaccgcg ccgagccctg ggatttcaag ctgccgcccg tgatgtggca cagccgctg 840
gccggccgcc ccgagaccga gggcgccgcc ctggtgtaa 879
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<210> SEQ ID NO 2
<211> LENGTH: 292
<212> TYPE: PRT
<213> ORGANISM: Delftia acidovorans
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```
<400> SEQUENCE: 2
```

```
Met Gln Thr Thr Leu Gln Ile Thr Pro Thr Gly Ala Thr Leu Gly Ala
1      5      10      15
Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala Gly Phe Ala
20     25     30
Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro Gly
35     40     45
Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe Gly
50     55     60
Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val Ala Ile Ser Asn Val
65     70     75     80
Lys Ala Asp Gly Thr Val Arg Gln His Ser Pro Ala Glu Trp Asp Asp
85     90     95
Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser Thr
100    105    110
Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val Val
115    120    125
Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala Tyr
130    135    140
Asp Ala Leu Asp Glu Ala Thr Arg Ala Leu Val His Gln Arg Ser Ala
145    150    155    160
Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln Gln
165    170    175
Ala Gly Ser Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr Pro
180    185    190
Leu Arg Pro Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser Leu
195    200    205
Leu Ile Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala Glu
210    215    220
Ser Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala Pro
225    230    235    240
Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp Asp
245    250    255
Asn Arg Cys Leu Leu His Arg Ala Glu Pro Trp Asp Phe Lys Leu Pro
260    265    270
Arg Val Met Trp His Ser Arg Leu Ala Gly Arg Pro Glu Thr Glu Gly
275    280    285
Ala Ala Leu Val
290
```

```
<210> SEQ ID NO 3
```

US 11,371,055 B2

111

112

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&lt;211&gt; LENGTH: 882

&lt;212&gt; TYPE: DNA

&lt;213&gt; ORGANISM: Delftia acidovorans

&lt;400&gt; SEQUENCE: 3

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atggctcaga ccactctcca aatcacaccc actggtgcc ccttgggtgc cacagtcact    60
gggtgttcacc ttgccacact tgacgatgct ggtttcgctg ccctccatgc agcctggcct    120
caacatgcac tcttgatott ccttgggcaa cacctcagca atgaccaaca gattacottt    180
gctaaacgct ttggagcaat tgagaggatt ggcggagggtg acattgttgc catatccaat    240
gtcaaggcag atggcacagt gcgccagcac tctcctgctg agtgggatga catgatgaag    300
gtcattgtgg gcaacatggc ctggcacgcc gactcaacct acatgccagt catggctcaa    360
ggagctgtgt tcagcgcaga agttgtccca gcagttgggg gcagaacctg ctttctgac    420
atgagggcag cctacgatgc ccttgatgag gcaacccgtg ctcttgttca ccaaaggctct    480
gctcgtcact cccttgtgta ttctcagagc aagttgggac atgtccaaca ggccgggtca    540
gcctacatag gttatggcat ggacaccact gcaactcctc tcagaccatt ggtaagggtg    600
catcctgaga ctggaaggcc cagcctcttg atcgcccgcc atgcccatgc catccctggc    660
atggatgcag ctgaatcaga gcgcttctct gaaggacttg ttgactgggc ctgccaggct    720
cccagagtcc atgctcacca atgggctgct ggagatgtgg ttgtgtggga caaccgtgt    780
ttgctccacc gtgctgagcc ctgggatttc aagttgccac gtgtgatgtg gcactccaga    840
ctcgtgggac gccagaaaac tgagggtgct gccttggttt ga                        882
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&lt;210&gt; SEQ ID NO 4

&lt;211&gt; LENGTH: 293

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Delftia acidovorans

&lt;400&gt; SEQUENCE: 4

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Met Ala Gln Thr Thr Leu Gln Ile Thr Pro Thr Gly Ala Thr Leu Gly
1      5      10      15
Ala Thr Val Thr Gly Val His Leu Ala Thr Leu Asp Asp Ala Gly Phe
20     25     30
Ala Ala Leu His Ala Ala Trp Leu Gln His Ala Leu Leu Ile Phe Pro
35     40     45
Gly Gln His Leu Ser Asn Asp Gln Gln Ile Thr Phe Ala Lys Arg Phe
50     55     60
Gly Ala Ile Glu Arg Ile Gly Gly Gly Asp Ile Val Ala Ile Ser Asn
65     70     75     80
Val Lys Ala Asp Gly Thr Val Arg Gln His Ser Pro Ala Glu Trp Asp
85     90     95
Asp Met Met Lys Val Ile Val Gly Asn Met Ala Trp His Ala Asp Ser
100    105    110
Thr Tyr Met Pro Val Met Ala Gln Gly Ala Val Phe Ser Ala Glu Val
115    120    125
Val Pro Ala Val Gly Gly Arg Thr Cys Phe Ala Asp Met Arg Ala Ala
130    135    140
Tyr Asp Ala Leu Asp Glu Ala Thr Arg Ala Leu Val His Gln Arg Ser
145    150    155    160
Ala Arg His Ser Leu Val Tyr Ser Gln Ser Lys Leu Gly His Val Gln
165    170    175
Gln Ala Gly Ser Ala Tyr Ile Gly Tyr Gly Met Asp Thr Thr Ala Thr
180    185    190
```

US 11,371,055 B2

113

114

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Pro Leu Arg Pro Leu Val Lys Val His Pro Glu Thr Gly Arg Pro Ser  
195 200 205

Leu Leu Ile Gly Arg His Ala His Ala Ile Pro Gly Met Asp Ala Ala  
210 215 220

Glu Ser Glu Arg Phe Leu Glu Gly Leu Val Asp Trp Ala Cys Gln Ala  
225 230 235 240

Pro Arg Val His Ala His Gln Trp Ala Ala Gly Asp Val Val Val Trp  
245 250 255

Asp Asn Arg Cys Leu Leu His Arg Ala Glu Pro Trp Asp Phe Lys Leu  
260 265 270

Pro Arg Val Met Trp His Ser Arg Leu Ala Gly Arg Pro Glu Thr Glu  
275 280 285

Gly Ala Ala Leu Val  
290

<210> SEQ ID NO 5  
<211> LENGTH: 882  
<212> TYPE: DNA  
<213> ORGANISM: Delftia acidovorans

<400> SEQUENCE: 5

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ggcgttcacc tggcgactct ggaatgacgca ggtttcgctg cgctgcacgc ggcttggtcg 120
caacatgctc tcttgatttt ccaggtcag cacctgtcca acgaccagca aatcactttt 180
gcaaaacgct tcggtgcgat cgaacgtatc ggtggcgggtg atattgtggc gatctccaac 240
gtaaaagcgg atggtactgt acgtcagcac agcccggcgg agtgggacga tatgatgaag 300
gtgatcgtag gcaacatggc atggcatgct gacagcacct acatgccggt tatggcgtag 360
ggtgcgggtt tctctgctga agtggttccg gcagtgggcg gtcgcacctg ctctgcagac 420
atgcgtgcag cttacgacgc gttagacgaa gctaccgcgc cactgggtaca ccagcgctct 480
gcgcgtcact ctctggtgta ttcccagagc aaactgggccc acgttcagca agcggggtcc 540
gcataatatc gctacggtat ggataccact gcgacccgcg tgcgtccgct ggtaaaagtg 600
catccggaaa ccggccgctc gtctctcctg atcggccgct acgctcatgc gattccgggt 660
atggacgcgg cagaatccga gcgtttcctg gaaggtctgg ttgattgggc ttgtcaggcg 720
ccgcgtgtgc atgctcacca gtgggcagct ggcgacgtgg ttgtatggga taaccgctgc 780
ctgcttcacc gtgcagaacc gtgggacttt aagctgccac gtgttatgtg gcacagccgt 840
ctggcaggcc gccagaaaac cgagggcgcg gctctggttt aa 882
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<210> SEQ ID NO 6  
<211> LENGTH: 16  
<212> TYPE: DNA  
<213> ORGANISM: Artificial sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Chemically synthesized M13 forward sequencing primer

<400> SEQUENCE: 6

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gtaaaacgac ggccag 16
```

<210> SEQ ID NO 7  
<211> LENGTH: 17  
<212> TYPE: DNA  
<213> ORGANISM: Artificial sequence  
<220> FEATURE:



US 11,371,055 B2

115

116

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<223> OTHER INFORMATION: Chemically synthesized M13 reverse sequencing primer

<400> SEQUENCE: 7

caggaaacag ctatgac 17

<210> SEQ ID NO 8

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: Chemically synthesized forward AAD-12 (v1) PTU primer

<400> SEQUENCE: 8

gaacagttag acatggtcta aagg 24

<210> SEQ ID NO 9

<211> LENGTH: 27

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: Chemically synthesized reverse AAD-12 (v1) PTU primer

<400> SEQUENCE: 9

gctgcaacac tgataaatgc caactgg 27

<210> SEQ ID NO 10

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: Chemically synthesized forward AAD-12 (v1) coding PCR primer

<400> SEQUENCE: 10

atggctcaga ccactctcca aa 22

<210> SEQ ID NO 11

<211> LENGTH: 20

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: Chemically synthesized reverse AAD-12 (v1) coding PCR primer

<400> SEQUENCE: 11

agctgcatcc atgccaggga 20

<210> SEQ ID NO 12

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: Chemically synthesized SdpacodF: AAD-12 (v1) forward coding region primer

<400> SEQUENCE: 12

atggctcatg ctgccctcag cc 22

<210> SEQ ID NO 13

<211> LENGTH: 22

<212> TYPE: DNA

<213> ORGANISM: Artificial sequence

<220> FEATURE:

<223> OTHER INFORMATION: Chemically synthesized SdpacodR: AAD-12 (v1)

US 11,371,055 B2

117

118

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reverse coding region primer

<400> SEQUENCE: 13

cgggcaggcc taactccacc aa 22

<210> SEQ ID NO 14  
<211> LENGTH: 32  
<212> TYPE: DNA  
<213> ORGANISM: Artificial sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Chemically synthesized primer "NcoI of Brady"

<400> SEQUENCE: 14

tataccacat gtcgatcgcc atccggcagc tt 32

<210> SEQ ID NO 15  
<211> LENGTH: 33  
<212> TYPE: DNA  
<213> ORGANISM: Artificial sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Chemically synthesized primer "SacI of Brady"

<400> SEQUENCE: 15

gagctcctat cactccgcgc cctgctgctg cac 33

<210> SEQ ID NO 16  
<211> LENGTH: 34  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer brjap 5' (speI)

<400> SEQUENCE: 16

actagtaaca aagaaggaga tataccatga cgat 34

<210> SEQ ID NO 17  
<211> LENGTH: 33  
<212> TYPE: DNA  
<213> ORGANISM: Artificial Sequence  
<220> FEATURE:  
<223> OTHER INFORMATION: Primer br jap 3' (xhoI)

<400> SEQUENCE: 17

ttctcgagct atcactccgc cgctgctgc tgc 33

<210> SEQ ID NO 18  
<211> LENGTH: 287  
<212> TYPE: PRT  
<213> ORGANISM: Ralstonia eutropha JMP134

<400> SEQUENCE: 18

Met Ser Val Val Ala Asn Pro Leu His Pro Leu Phe Ala Ala Gly Val  
1 5 10 15

Glu Asp Ile Asp Leu Arg Glu Ala Leu Gly Ser Thr Glu Val Arg Glu  
20 25 30

Ile Glu Arg Leu Met Asp Glu Lys Ser Val Leu Val Phe Arg Gly Gln  
35 40 45

Pro Leu Ser Gln Asp Gln Gln Ile Ala Phe Ala Arg Asn Phe Gly Pro  
50 55 60

Leu Glu Gly Gly Phe Ile Lys Val Asn Gln Arg Pro Ser Arg Phe Lys  
65 70 75 80

Tyr Ala Glu Leu Ala Asp Ile Ser Asn Val Ser Leu Asp Gly Lys Val  
85 90 95

US 11,371,055 B2

119

120

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Ala Gln Arg Asp Ala Arg Glu Val Val Gly Asn Phe Ala Asn Gln Leu  
100 105 110  
Trp His Ser Asp Ser Ser Phe Gln Gln Pro Ala Ala Arg Tyr Ser Met  
115 120 125  
Leu Ser Ala Val Val Val Pro Pro Ser Gly Gly Asp Thr Glu Phe Cys  
130 135 140  
Asp Met Arg Ala Ala Tyr Asp Ala Leu Pro Arg Asp Leu Gln Ser Glu  
145 150 155 160  
Leu Glu Gly Leu Arg Ala Glu His Tyr Ala Leu Asn Ser Arg Phe Leu  
165 170 175  
Leu Gly Asp Thr Asp Tyr Ser Glu Ala Gln Arg Asn Ala Met Pro Pro  
180 185 190  
Val Asn Trp Pro Leu Val Arg Thr His Ala Gly Ser Gly Arg Lys Phe  
195 200 205  
Leu Phe Ile Gly Ala His Ala Ser His Val Glu Gly Leu Pro Val Ala  
210 215 220  
Glu Gly Arg Met Leu Leu Ala Glu Leu Leu Glu His Ala Thr Gln Arg  
225 230 235 240  
Glu Phe Val Tyr Arg His Arg Trp Asn Val Gly Asp Leu Val Met Trp  
245 250 255  
Asp Asn Arg Cys Val Leu His Arg Gly Arg Arg Tyr Asp Ile Ser Ala  
260 265 270  
Arg Arg Glu Leu Arg Arg Ala Thr Thr Leu Asp Asp Ala Val Val  
275 280 285

&lt;210&gt; SEQ ID NO 19

&lt;211&gt; LENGTH: 289

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Bradyrhizobium japonicum USDA 110

&lt;400&gt; SEQUENCE: 19

Met Thr Ile Ala Ile Arg Gln Leu Gln Thr His Phe Val Gly Gln Val  
1 5 10 15  
Ser Gly Leu Asp Leu Arg Lys Pro Leu Thr Pro Gly Glu Ala Arg Glu  
20 25 30  
Val Glu Ser Ala Met Asp Lys Tyr Ala Val Leu Val Phe His Asp Gln  
35 40 45  
Asp Ile Thr Asp Glu Gln Gln Met Ala Phe Ala Leu Asn Phe Gly Gln  
50 55 60  
Arg Glu Asp Ala Arg Gly Gly Thr Val Thr Lys Glu Lys Asp Tyr Arg  
65 70 75 80  
Leu Gln Ser Gly Leu Asn Asp Val Ser Asn Leu Gly Lys Asp Gly Lys  
85 90 95  
Pro Leu Ala Lys Asp Ser Arg Thr His Leu Phe Asn Leu Gly Asn Cys  
100 105 110  
Leu Trp His Ser Asp Ser Ser Phe Arg Pro Ile Pro Ala Lys Phe Ser  
115 120 125  
Leu Leu Ser Ala Arg Val Val Asn Pro Thr Gly Gly Asn Thr Glu Phe  
130 135 140  
Ala Asp Met Arg Ala Ala Tyr Asp Ala Leu Asp Asp Glu Thr Lys Ala  
145 150 155 160  
Glu Ile Glu Asp Leu Val Cys Glu His Ser Leu Met Tyr Ser Arg Gly  
165 170 175  
Ser Leu Gly Phe Thr Glu Tyr Thr Asp Glu Glu Lys Gln Met Phe Lys

US 11,371,055 B2

121

122

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180					185					190					
Pro	Val	Leu	Gln	Arg	Leu	Val	Arg	Thr	His	Pro	Val	His	Arg	Arg	Lys
	195						200					205			
Ser	Leu	Tyr	Leu	Ser	Ser	His	Ala	Gly	Lys	Ile	Ala	Ser	Met	Ser	Val
	210					215					220				
Pro	Glu	Gly	Arg	Leu	Leu	Leu	Arg	Asp	Leu	Asn	Glu	His	Ala	Thr	Gln
	225					230					235				240
Pro	Glu	Phe	Val	Tyr	Val	His	Lys	Trp	Lys	Leu	His	Asp	Leu	Val	Met
			245						250					255	
Trp	Asp	Asn	Arg	Gln	Thr	Met	His	Arg	Val	Arg	Arg	Tyr	Asp	Gln	Ser
			260					265					270		
Gln	Pro	Arg	Asp	Met	Arg	Arg	Ala	Thr	Val	Ala	Gly	Thr	Glu	Pro	Thr
		275					280					285			

Val

&lt;210&gt; SEQ ID NO 20

&lt;211&gt; LENGTH: 295

&lt;212&gt; TYPE: PRT

&lt;213&gt; ORGANISM: Sphingobium herbicidovorans

&lt;400&gt; SEQUENCE: 20

Met	His	Ala	Ala	Leu	Ser	Pro	Leu	Ser	Gln	Arg	Phe	Glu	Arg	Ile	Ala
1				5					10					15	
Val	Gln	Pro	Leu	Thr	Gly	Val	Leu	Gly	Ala	Glu	Ile	Thr	Gly	Val	Asp
			20					25					30		
Leu	Arg	Glu	Pro	Leu	Asp	Asp	Ser	Thr	Trp	Asn	Glu	Ile	Leu	Asp	Ala
		35				40					45				
Phe	His	Thr	Tyr	Gln	Val	Ile	Tyr	Phe	Pro	Gly	Gln	Ala	Ile	Thr	Asn
	50					55					60				
Glu	Gln	His	Ile	Ala	Phe	Ser	Arg	Arg	Phe	Gly	Pro	Val	Asp	Pro	Val
	65			70					75					80	
Pro	Leu	Leu	Lys	Ser	Ile	Glu	Gly	Tyr	Pro	Glu	Val	Gln	Met	Ile	Arg
			85					90						95	
Arg	Glu	Ala	Asn	Glu	Ser	Gly	Arg	Val	Ile	Gly	Asp	Asp	Trp	His	Thr
		100					105						110		
Asp	Ser	Thr	Phe	Leu	Asp	Ala	Pro	Pro	Ala	Ala	Val	Val	Met	Arg	Ala
		115				120						125			
Ile	Asp	Val	Pro	Glu	His	Gly	Gly	Asp	Thr	Gly	Phe	Leu	Ser	Met	Tyr
	130					135					140				
Thr	Ala	Trp	Glu	Thr	Leu	Ser	Pro	Thr	Met	Gln	Ala	Thr	Ile	Glu	Gly
	145				150				155					160	
Leu	Asn	Val	Val	His	Ser	Ala	Thr	Arg	Val	Phe	Gly	Ser	Leu	Tyr	Gln
			165					170						175	
Ala	Gln	Asn	Arg	Arg	Phe	Ser	Asn	Thr	Ser	Val	Lys	Val	Met	Asp	Val
		180					185						190		
Asp	Ala	Gly	Asp	Arg	Glu	Thr	Val	His	Pro	Leu	Val	Val	Thr	His	Pro
	195						200					205			
Gly	Ser	Gly	Arg	Lys	Gly	Leu	Tyr	Val	Asn	Gln	Val	Tyr	Cys	Gln	Arg
	210					215					220				
Ile	Glu	Gly	Met	Thr	Asp	Ala	Glu	Ser	Lys	Pro	Leu	Leu	Gln	Phe	Leu
	225				230				235					240	
Tyr	Glu	His	Ala	Thr	Arg	Phe	Asp	Phe	Thr	Cys	Arg	Val	Arg	Trp	Lys
		245						250					255		
Lys	Asp	Gln	Val	Leu	Val	Trp	Asp	Asn	Leu	Cys	Thr	Met	His	Arg	Ala

US 11,371,055 B2

123

124

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260	265	270
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20	25	30
Glu Gln Leu Tyr His Ala Val Leu Arg His Gln Val Val Phe Leu Arg		
35	40	45
Asp Gln Ala Ile Thr Pro Gln Gln Gln Arg Ala Leu Ala Gln Arg Phe		
50	55	60
Gly Glu Leu His Ile His Pro Val Tyr Pro His Ala Glu Gly Val Asp		
65	70	75 80
Glu Ile Ile Val Leu Asp Thr His Asn Asp Asn Pro Pro Asp Asn Asp		
85	90	95
Asn Trp His Thr Asp Val Thr Phe Ile Glu Thr Pro Pro Ala Gly Ala		
100	105	110
Ile Leu Ala Ala Lys Glu Leu Pro Ser Thr Gly Gly Asp Thr Leu Trp		
115	120	125
Thr Ser Gly Ile Ala Ala Tyr Glu Ala Leu Ser Val Pro Phe Arg Gln		
130	135	140
Leu Leu Ser Gly Leu Arg Ala Glu His Asp Phe Arg Lys Ser Phe Pro		
145	150	155 160
Glu Tyr Lys Tyr Arg Lys Thr Glu Glu Glu His Gln Arg Trp Arg Glu		
165	170	175
Ala Val Ala Lys Asn Pro Pro Leu Leu His Pro Val Val Arg Thr His		
180	185	190
Pro Val Ser Gly Lys Gln Ala Leu Phe Val Asn Glu Gly Phe Thr Thr		
195	200	205
Arg Ile Val Asp Val Ser Glu Lys Glu Ser Glu Ala Leu Leu Ser Phe		
210	215	220
Leu Phe Ala His Ile Thr Lys Pro Glu Phe Gln Val Arg Trp Arg Trp		
225	230	235 240
Gln Pro Asn Asp Ile Ala Ile Trp Asp Asn Arg Val Thr Gln His Tyr		
245	250	255
Ala Asn Ala Asp Tyr Leu Pro Gln Arg Arg Ile Met His Arg Ala Thr		
260	265	270
Ile Leu Gly Asp Lys Pro Phe Tyr Arg Ala Gly		
275	280	

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US 11,371,055 B2

125

We claim:

1. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-12 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and a pyridyloxy auxin herbicide, further wherein said AAD-12 protein comprises:

- i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 2; and
- ii) an AAD-12 motif having the general formula of:

$HX_{109}D(X)_{111-134}T(X)_{136-261}H(X)_{263-272}R$ , wherein

$X_{109}$  represents a single amino acid at position 109, relative to the sequence of SEQ ID NO: 2;

$(X)_{111-134}$  represents a sequence of 24 amino acids;

$(X)_{136-261}$  represents a sequence of 126 amino acids; and

$(X)_{263-272}$  represents a sequence of 10 amino acids.

2. The plant cell of claim 1 wherein the AAD-12 protein comprises an amino acid sequence having at least 90% sequence identity with SEQ ID NO: 2.

3. The plant cell of claim 1 wherein said plant cell is dicotyledonous and selected from the group consisting of a cotton cell, a tobacco cell, a canola cell, a soybean cell, and an *Arabidopsis* cell.

4. A transgenic plant comprising a plurality of the plant cell of claim 1, wherein expression of said polynucleotide renders said plant tolerant to an aryloxyalkanoate herbicide.

5. The plant of claim 4 wherein said aryloxyalkanoate herbicide is a phenoxy auxin herbicide.

6. The plant of claim 4 wherein said aryloxyalkanoate herbicide is selected from the group consisting of 2,4-dichlorophenoxyacetic acid, and MCPA.

7. The plant of claim 4 wherein said aryloxyalkanoate herbicide is a pyridyloxy auxin.

8. The plant of claim 4 wherein said aryloxyalkanoate herbicide is selected from the group consisting of triclopyr and fluroxypyr.

9. The plant of claim 4 wherein expression of said polynucleotide renders said plant resistant to both a phenoxy auxin herbicide and a pyridyloxy auxin herbicide.

10. The plant of claim 4 wherein said plant further comprises a second herbicide resistance gene.

11. The plant of claim 10 wherein said second herbicide resistance gene renders said plant resistant to an herbicide selected from the group consisting of glyphosate, glufosinate, ALS inhibitors, inhibitors of 4-hydroxyphenyl-pyruvate-dioxygenase (HPPD), dicamba and inhibitors of protoporphyrinogen oxidase (PPO).

12. A method of controlling at least one weed in a field, wherein said field contains at least one plant of claim 4, wherein said method comprises applying to at least a portion of said field a first herbicide selected from the group consisting of a phenoxy auxin herbicide and a pyridyloxy auxin herbicide.

13. The method of claim 12 wherein said aryloxyalkanoate herbicide is an achiral phenoxy auxin selected from the group consisting of 2,4-D and MCPA.

14. The method of claim 12 wherein said aryloxyalkanoate herbicide is a pyridyloxy auxin selected from the group consisting of triclopyr and fluroxypyr.

15. The method of claim 12 wherein said method comprises applying a second herbicide.

16. The method of claim 15 wherein said first herbicide and said second herbicide are applied sequentially.

17. The method of claim 15 wherein said first herbicide and said second herbicide are applied concurrently.

126

18. The method of claim 15 wherein said first herbicide is a phenoxy auxin and said second herbicide is a pyridyloxy auxin.

19. The method of claim 15 wherein said second herbicide is selected from the group consisting of glyphosate, glufosinate, dicamba, acetolactate synthase inhibitors, protoporphyrinogen oxidase inhibitors, and hydroxyphenyl-pyruvate-dioxygenase inhibitors.

20. The method of claim 15, wherein said first herbicide is 2,4-D and said second herbicide is glyphosate.

21. The method of claim 15, wherein said first herbicide is 2,4-D and said second herbicide is glufosinate.

22. The method of claim 15 wherein said plant further comprises a second herbicide resistance gene that renders said plant resistant to said second herbicide.

23. The method of claim 22 wherein said second herbicide resistance gene is selected from the group consisting of a modified AHAS (acetohydroxyacid synthase) gene, a glyphosate resistance gene, glufosinate resistance gene, and a gene encoding a dicamba-degrading enzyme.

24. The method of claim 23 wherein:

(a) said modified AHAS (acetohydroxyacid synthase) gene is selected from the group consisting of SurA, SurB, Csr1, Csr1-1, and Csr1-2;

(b) said glyphosate resistance gene is selected from the group consisting of modified EPSPS (5-enolpyruvyl-shikimate-3-phosphate synthase), GOX, and GAT; and, said glufosinate resistance gene is selected from the group consisting of phosphinothricin-N-acetyltransferase (PAT) and bar.

25. The method of claim 15 wherein said method further comprises applying a third herbicide.

26. The method of claim 25, wherein said third herbicide is selected from the group consisting of glyphosate, glufosinate, HPPD-inhibitors, PPO-inhibitors, ALS inhibitors, and dicamba.

27. The method of claim 26 wherein said first, second and third herbicides are 2,4-D, quizalofop, and glyphosate.

28. A seed comprising a plurality of the plant cell of claim 1.

29. A method of controlling weeds in a field, wherein said method comprises applying a phenoxy auxin herbicide or a pyridyloxy auxin herbicide to said field and planting a seed of claim 28 in said field within 14 days after applying said aryloxyalkanoate or pyridyloxy auxin herbicide.

30. A plant grown from the seed of claim 28 wherein said plant comprises said polynucleotide.

31. A part, progeny, or asexual propagate of the plant of claim 30, wherein said part, progeny, or sexual propagate comprises said polynucleotide.

32. A transgenic plant cell comprising a recombinant polynucleotide that encodes an AAD-12 protein that exhibits aryloxyalkanoate dioxygenase activity wherein said activity enzymatically degrades a phenoxy auxin herbicide and a pyridyloxy auxin herbicide, further wherein said AAD-12 protein comprises:

- i) an amino acid sequence having at least 85% sequence identity with SEQ ID NO: 2; and
- ii) an AAD-12 motif having the general formula of:

$HX_{109}D(X)_{111-134}T(X)_{136-261}H(X)_{263-272}R$ , wherein

$X_{109}$  represents a single amino acid at position 109, relative to the sequence of SEQ ID NO: 2;

$(X)_{111-134}$  represents a sequence of 24 amino acids;

$(X)_{136-261}$  represents a sequence of 126 amino acids; and



US 11,371,055 B2

127

128

(X)<sub>263-272</sub> represents a sequence of 10 amino acids,  
wherein said

AAD-12 motif has 90% sequence identity with correspond-  
ing amino acids of position 108 to 273 of SEQ ID NO: 2.

33. A method of controlling at least one weed in a field, 5  
wherein said field has been planted with seeds wherein cells  
of said seeds comprise

a recombinant polynucleotide that encodes an AAD-12  
protein that exhibits aryloxyalkanoate dioxygenase  
activity wherein said activity enzymatically degrades a 10  
phenoxy auxin herbicide and a pyridyloxy auxin her-  
bicide, further wherein said AAD-12 protein com-  
prises:

- i) an amino acid sequence having at least 85% sequence  
identity with SEQ ID NO: 2; and 15
- ii) an AAD-12 motif having the general formula of:

$HX_{109}D(X)_{111-134}T(X)_{136-261}H(X)_{263-272}R$ , wherein

$X_{109}$  represents a single amino acid at position 109,  
relative to the sequence of SEQ ID NO: 2; 20

$(X)_{111-134}$  represents a sequence of 24 amino acids;

$(X)_{136-261}$  represents a sequence of 126 amino acids;  
and

$(X)_{263-272}$  represents a sequence of 10 amino acids,  
wherein said method comprises applying to said field a 25  
pyridyloxy auxin herbicide.

\* \* \* \* \*

# **Exhibit R**



UNITED STATES PATENT AND TRADEMARK OFFICE

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/559,177	07/26/2012	Nathan Bard	DAS-P0292-01-US	6091
83067	7590	07/10/2013		
Faegre Baker Daniels LLP- Dow AgroSciences 300 North Meridian Street, Suite 2700 Indianapolis, IN 46204			EXAMINER KOVALENKO, MYKOLA V	
			ART UNIT	PAPER NUMBER
			1638	
			NOTIFICATION DATE	DELIVERY MODE
			07/10/2013	ELECTRONIC

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

inteas@faegrebd.com  
cynthia.payson@faegrebd.com

Application No.  
13/559,177Applicant(s)  
BARD ET AL.**Office Action Summary**Examiner  
MYKOLA KOVALENKOArt Unit  
1638AIA (First Inventor to File)  
Status  
No**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --****Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

- 1) ☒ Responsive to communication(s) filed on 26 July 2012.  
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on \_\_\_\_.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on \_\_\_\_; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims**

- 5) ☒ Claim(s) 1-12 is/are pending in the application.  
 5a) Of the above claim(s) \_\_\_\_ is/are withdrawn from consideration.
- 6) ☐ Claim(s) \_\_\_\_ is/are allowed.
- 7) ☒ Claim(s) 1-12 is/are rejected.
- 8) ☐ Claim(s) \_\_\_\_ is/are objected to.
- 9) ☐ Claim(s) \_\_\_\_ are subject to restriction and/or election requirement.

\* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see [http://www.uspto.gov/patents/init\\_events/pph/index.jsp](http://www.uspto.gov/patents/init_events/pph/index.jsp) or send an inquiry to [PPHfeedback@uspto.gov](mailto:PPHfeedback@uspto.gov).

**Application Papers**

- 10) ☒ The specification is objected to by the Examiner.
- 11) ☒ The drawing(s) filed on 26 July 2012 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.  
 Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
 Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

- a) ☐ All b) ☐ Some \* c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

- 1) ☒ Notice of References Cited (PTO-892)
- 2) ☐ Information Disclosure Statement(s) (PTO/SB/08)  
 Paper No(s)/Mail Date \_\_\_\_.
- 3) ☐ Interview Summary (PTO-413)  
 Paper No(s)/Mail Date \_\_\_\_.
- 4) ☐ Other: \_\_\_\_.

Application/Control Number: 13/559,177  
Art Unit: 1638

Page 2

### **DETAILED ACTION**

1. Claims 1-12 are pending.
2. Claims 1-12 are examined herein.

### ***Claim Objections***

3. Claims 1, 5, 7, and 10 are objected to because of the following informalities:

At claim 1, line 1, the phrase “a method of controlling insects that comprises” should be replaced with “a method of controlling insects, said method comprising,” for clarity.

Similarly, at claim 5, line 1, the phrase “that comprises” should be replaced with “said method comprising.”

At claims 1 and 5, last line of each claim, the phrase “diagnostic for the presence” should be changed to “diagnostic of the presence,” because the latter reflects proper syntax.

At claim 7, line 1, the term “comprising” should be changed to “said method comprising,” to clarify that the subsequent recitation refers to the method and not to a soybean plant. At claim 7, lines 6-7, the phrase “for presence of DNA” requires the definite article “the” before “presence.”

At claim 10, line 2, the phrase “identity with SEQ ID NO: 14” should be changed to “identity to SEQ ID NO: 14” because the latter usage is the art-recognized convention. At claim 10, line 3, the phrase “American Type Culture Collection” requires the definite article “the” before “American.” At claim 10, line 2, the phrase “soybean

Application/Control Number: 13/559,177

Page 3

Art Unit: 1638

plant has been deposited” should be changed to “soybean plant, wherein representative seed of said soybean plant has been deposited” to properly reflect the deposit statement (see pg. 2, paragraph 6 of the specification).

Appropriate correction is required.

### ***Specification***

4. The disclosure is objected to because in lines 1-3 of paragraph 1, Applicant claims priority to US Provisional Applications No. 61/511,664 and 61/521,798. Applicant cannot claim priority to a provisional application, only “benefit of” a provisional application.

The abstract is objected to because in line 1, the phrase “Soybean event 9582.814.19.1 comprises genes encoding” is grammatically incorrect. The phrase should either have the word “comprises” replaced with “comprising,” or the words “wherein the event comprises” inserted after “9582.814.19.1.”

Appropriate correction is required.

### ***Claim Rejections - 35 USC § 112 - Second Paragraph***

5. The following is a quotation of 35 U.S.C. 112(b):

(B) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:

The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.



Application/Control Number: 13/559,177  
Art Unit: 1638

Page 4

6. Claims 7-12 are rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

At claims 7, 8, and 12, last line of each claim, the term “complements thereof” is unclear. The disclosure does not define the term “complement” and its precise meaning would not be immediately apparent to one skilled in the art. For example, one could interpret the term to encompass both, a partial and a full complement at the same time. The metes and bounds of the claims are unclear. Because claims 9-11 depend from claim 8, they are indefinite as well.

At claim 11, line 1, the term “derived” is unclear. The specification does not define the term and one could interpret “derivation” as either preserving the claimed event 9582.814.19.1 in the “derived” composition or not. The metes and bounds of the claim are unclear.

At claim 12, line 2, the term “including soybean event” is unclear. The specification does not define the term “including” in the context of “including” a soybean event into grain, seed, meal, or flour, and its meaning would not be readily apparent to one skilled in the art. The metes and bounds of the claim are thus unclear.

A broad range or limitation together with a narrow range or limitation that falls within the broad range or limitation (in the same claim) is considered indefinite, since the resulting claim does not clearly set forth the metes and bounds of the patent protection desired. See MPEP § 2173.05(c). Note the explanation given by the Board

Application/Control Number: 13/559,177

Page 5

Art Unit: 1638

of Patent Appeals and Interferences in *Ex parte Wu*, 10 USPQ2d 2031, 2033 (Bd. Pat. App. & Inter. 1989), as to where broad language is followed by "such as" and then narrow language. The Board stated that this can render a claim indefinite by raising a question or doubt as to whether the feature introduced by such language is (a) merely exemplary of the remainder of the claim, and therefore not required, or (b) a required feature of the claims. Note also, for example, the decisions of *Ex parte Steigewald*, 131 USPQ 74 (Bd. App. 1961); *Ex parte Hall*, 83 USPQ 38 (Bd. App. 1948); and *Ex parte Hasche*, 86 USPQ 481 (Bd. App. 1949). In the present instance, claim 10 recites the broad recitation "the soybean plant ... comprising a DNA sequence having at least 95% sequence identity with SEQ ID NO: 14," and the claim also recites "soybean plant ... deposited with American Type Culture Collection under Accession No. PTA-12006," which is the narrower statement of the range/limitation. The instant specification teaches that the soybean plants of the recited accession number comprise the full-length SEQ ID NO: 14 without substitutions and thus the recitation of a "DNA sequence having at least 95% sequence identity with SEQ ID NO: 14" is broader in scope.

### ***Claim Rejections - 35 USC § 112 - Written Description***

7. The following is a quotation of 35 U.S.C. 112(a):

(a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to

Application/Control Number: 13/559,177

Page 6

Art Unit: 1638

enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

8. Claims 1-5 and 7-12 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor or a joint inventor, or for pre-AIA the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicant claims a method of controlling any insects using a soybean plant that comprises at least one of the recited fragments of SEQ ID NO: 1 or 2, said fragments being diagnostic of the presence of event 9582.814.19.1 (instant claims 1-5). Applicant claims a method of breeding a soybean plant said method comprising assaying the resulting plant for the presence of at least one of the recited fragments of SEQ ID NO: 1 and 2, or complements thereof (instant claim 7). Applicant claims a soybean plant or part thereof that is resistant to soybean looper and comprises at least one of the recited fragments of SEQ ID NO: 1 and 2, or complements thereof; Applicant claims the seed of said plant, and a composition derived from said plant (instant claims 8-9, 11). Applicant claims a soybean plant or part thereof comprising a DNA sequence having at least 95% sequence identity to the instant SEQ ID NO: 14, wherein said soybean plant has been deposited with the ATCC under Accession No. PTA-12006 (instant claim 10), Applicant claims a method of controlling pests in soybean grain, seed, meal or flour, which

Application/Control Number: 13/559,177

Page 7

Art Unit: 1638

comprise the recited fragments of SEQ ID NO: 1 and 2, or complements thereof (instant claim 12).

Applicant describes a soybean plant comprising soybean event 9582.814.19.1 (Example 1, beginning on pg. 30; Example 2, beginning on pg. 31 of the specification). Applicant describes making said plant by transforming a soybean plant with the plasmid pDAB9582 comprising the PAT gene as well as the genes encoding Cry1F and Cry1Ac proteins (Example 1, beginning on pg. 30 of the specification). Applicant describes the representative seed of event 9582.814.19.1 that has been deposited with the ATCC under ATCC Seed Deposit PTA-12006 (pg. 6, paragraph 22 of the specification). Applicant describes the sequences of foreign and flanking DNA comprising event DP-9582.814.19.1 (SEQ ID NO: 14 representing the entire event), which includes the coding sequences of the PAT gene, Cry1F, and Cry1Ac genes, and the border sequences of SEQ ID NO: 1 and 2 (pg. 7-8, Brief Description of the Sequences).

Applicant does not describe the claimed genus of any other soybean plants comprising a DNA sequence selected from the group of the regions of SEQ ID NO: 1 or 2, recited in claim 1, for example (instant claims 1-5 and 7-12). Applicant does not describe a genus of undefined complements of any of the recited regions, which complements would read on any di-nucleotide (instant claims 7, 8). Applicant does not describe any soybean plant comprising a DNA sequence having at least 95% sequence identity to the instant SEQ ID NO: 14, which sequence represents the full sequence of the claimed event (instant claim 10). For example, SEQ ID NO: 14 is 15,294 nucleotides long. A nucleotide sequence with 95% sequence identity to it could

Application/Control Number: 13/559,177

Page 8

Art Unit: 1638

comprise up to 765 substitutions anywhere along the length of the sequence. Applicant does not describe this vast genus of sequences. In addition, Applicant does not describe soybean plants resistant to any insects besides the three species recited in claims 2-4. As is well known in the art, individual Cry proteins confer resistance to only a limited number of insects or nematodes. For example, deMaagd et al teach that “Individual Cry toxins have a defined spectrum of insecticidal activity, usually restricted to a few species within one particular order of insects” (deMaagd et al, pg. 193, right col., first full paragraph). Thus having described soybean plants resistant to three species of lepidopteran insects (due to Cry protein transgenes), Applicant cannot validly claim to have described soybean plants resistant to any insect pests other than soybean looper, velvet bean caterpillar, and fall armyworm. Because a method is not described if the products used in the method are not described, and because Applicant has not described the above plants, Applicant has not described the methods of making or using them either, nor any compositions derived therefrom (instant claims 1-5, 7, 11, 12).

For these reasons, it is unclear whether Applicant was in possession of the invention as broadly claimed.

### ***Claim Rejections - 35 USC § 112 - Enablement***

9. The following is a quotation of 35 U.S.C. 112(a):  
(a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

Application/Control Number: 13/559,177  
Art Unit: 1638

Page 9

The following is a quotation of 35 U.S.C. 112 (pre-AIA), first paragraph:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

10. Claims 1-5 and 7-12 are rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, because the specification, while being enabling for a soybean plant of event 9582.814.19.1, the representative seed of which was deposited with the ATCC under Accession No. PTA-12006, and for the methods of using those plants to control weeds using glufosinate, and to control three species of coleopteran insects (velvet bean caterpillar, soybean looper, and fall armyworm); and while being enabling for the progeny of the deposited line, which progeny retains the event (as defined by the presence of the transgene and a diagnostic fragment of SEQ ID NO: 1 in addition to a diagnostic fragment of SEQ ID NO: 2), does not reasonably provide enablement for the genus of any soybean plant comprising SEQ ID NO: 1 or 2 or their fragments or complements; or method of using and making said plant. The specification does not reasonably provide enablement for using said plants to control any insects or any pests, besides said three species of insects. Nor does the specification reasonably provide enablement for any seed of such plant, or any composition derived from such plant. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the invention commensurate in scope with these claims.



Application/Control Number: 13/559,177  
Art Unit: 1638

Page 10

*In re Wands*, 858 F.2d 731 (Fed. Cir. 1988) lists the following eight factors for determining whether undue experimentation would be required to practice an invention: (1) quantity of experimentation necessary; (2) amount of direction or guidance supplied; (3) presence or absence of working examples; (4) nature of the invention; (5) state of the prior art; (6) relative skill of those in the art; (7) predictability or unpredictability of the prior art; (8) breadth of the claims.

Applicant broadly claims a method of controlling any insects using a soybean plant that comprises at least one of the recited fragments of SEQ ID NO: 1 or 2, said fragments being diagnostic of the presence of event 9582.814.19.1 (instant claims 1-5). Applicant claims a method of breeding a soybean plant said method comprising assaying the resulting plant for the presence of at least of the recited fragments of SEQ ID NO: 1 and 2, or complements thereof (instant claim 7). Applicant claims a soybean plant or part thereof that is resistant to soybean looper and comprises at least one of the recited fragments of SEQ ID NO: 1 and 2, or complements thereof; Applicant claims the seed of said plant, and a composition derived from said plant (instant claims 8-9, 11). Applicant claims a soybean plant or part thereof comprising a DNA sequence having at least 95% sequence identity to the instant SEQ ID NO: 14, wherein said soybean plant has been deposited with the ATCC under Accession No. PTA-12006 (instant claim 10), Applicant claims a method of controlling any pests in soybean grain, seed, meal or flour, which comprise the recited fragments of SEQ ID NO: 1 and 2, or complements thereof (instant claim 12).

Application/Control Number: 13/559,177  
Art Unit: 1638

Page 11

Applicant teaches how to transform soybean plants with the DNA construct pDAB9582 that comprises the PAT gene, Cry1F and Cry1Ac genes, and how to identify the presence of the resulting transformation event 9582.814.19.1 (represented by the full-length SEQ ID NO: 14) in soybean plants (Example 1 on pg. 30; Example 2 on pg. 31; Example 3 on pg. 33 of the specification). Applicant teaches that the representative seeds of the event were deposited with the ATCC under Accession No. PTA-12006 (pg. 6, paragraph 22 of the specification). Applicant teaches the nucleic acid sequences that comprise the transgene gene, which sequences, along with the flanking border regions (SEQ ID NO: 1 and 2), comprise event 9582.814.19.1, represented by the full-length SEQ ID NO: 14, which encompasses SEQ ID NO: 1 and 2. Applicant thus teaches the progeny of the plants, the representatives seeds of which have been deposited under Accession No. PTA-12006, which progeny comprises the transgene and the fragments of SEQ ID NO: 1 and 2, which fragments are diagnostic of event 9582.814.19.1. Applicant teaches that the regions of the flanking regions SEQ ID NO: 1 and 2, which regions are recited in the instant claim 1, for example, represent the junction regions between the genomic DNA of the soybean and the DNA of the transgene (Brief Description of The Sequences on pg. 7, paragraphs 23-24).

Applicant does not teach how to make or use a genus of any other soybean plant that comprises a DNA sequence of the flanking regions (SEQ ID NO: 1 and 2), any of their regions or any complements thereof (instant claims 1-5, 7-9, 11-12). Applicant does not teach how to make or use a genus of any soybean plant or any part thereof comprising a DNA sequence that has 95% sequence identity to the instant SEQ ID NO:

Application/Control Number: 13/559,177

Page 12

Art Unit: 1638

14 (instant claim 10). Applicant does not teach how to use said plants to control any insects other than the three species listed above (instant claim 1), or how to control any pests in a soybean grain, seed, meal or flour comprising “including” soybean event into said gran, seed, meal or flour, other than soybean looper, velvet bean caterpillar, and fall armyworm (instant claim 12).

First, as detailed in the immediately preceding section, it is well-known in the art that the range of the species of pests against which individual Cry proteins are active is narrow and limited to only a few species (see DeMaagd et al, pg. 193, right col., first full paragraph). Next, Applicant's own teachings and the prior art indicate that the enablement of the present invention is limited to the soybean plants grown from the seeds deposited under ATCC Seed Deposit PTA-12006, which seed comprises event 9582.814.19.1 and to the methods involving only those plants. The disclosure teaches that “it is often necessary to screen a large number of events in order to identify an event characterized by optimal expression of the introduced gene of interest” (pg. 1, paragraph 3 of the specification).

The disclosure teaches that the integration site contains unique “junctions,” which are the regions at which the insert DNA joins the native plant's genome (pg. 10, paragraph 47 of the specification). The disclosure teaches that the instant SEQ ID NO: 1 and 2 are two examples of the junction DNA from event 9582.814.19.1 (pg. 7-8, Brief Description of the Sequences). These teachings do not appear to permit any flexibility to the sequences comprising the event and thus the claimed plants. For example, the event, as taught by the specification, cannot comprise a genus of polynucleotides with

Application/Control Number: 13/559,177

Page 13

Art Unit: 1638

95% sequence identity to the DNA of the event, which is SEQ ID NO: 14 in the instant case. This means, for example, that the instant deposit of the representative number of seeds of the claimed event under Accession No. PTA-12006, does not enable soybean plants comprising DNA sequences with 95% sequence identity to the instant SEQ ID NO: 14, because the deposited seed is taught as comprising the full-length SEQ ID NO: 14, without the 5% of its nucleotides altered.

The state of the art teaches that it would be highly unpredictable to reproduce a transformation event in plants, because no efficient gene targeting system is available for flowering plants. Hohe et al, for example, teach that although great efforts have been undertaken to establish a gene targeting system in plants, to day, all attempts have been unsuccessful, except in one moss species (Hohe et al, Plant Cell Rep. (2003) 21:1135-1142, pg. 1135, right col.). Consequently, the only reproducible way to arrive at the insect and herbicide resistant soybean plants of the instant invention is to use the soybean plant, the representative seed of which having been deposited with the ATCC under Accession No. 12006 as a starting material to produce the progeny, which progeny retains the entire event 9582.814.19.1 (including the transgene and the flanking sequences).

Given limited guidance supplied by Applicant, the breadth of the claims and the nature of the invention, as well as the unpredictability in the art, it would have required one skilled in the art undue trial and error experimentation to practice the claimed invention.

Application/Control Number: 13/559,177  
Art Unit: 1638

Page 14

11. Claim 10 is rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The claim is directed to novel plants. Since the plants are essential to the claimed invention, they must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plants are not so obtainable or available, a deposit of the plant may satisfy the requirements of 35 USC 112. A deposit of 2,500 seeds of each of the claimed embodiments is considered sufficient to ensure public availability. The specification does not disclose a repeatable process to obtain the plants and it is not apparent whether the plants are readily available to the public. It is noted that Applicants have deposited the plants with the American Type Tissue Culture Collection under Accession Number PTA-12006, but there is no affirmative statement as to irrevocable public availability (see pg. 6, paragraph 22 for the Deposit Statement).

If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicants, or a statement by an attorney of record over his or her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

Application/Control Number: 13/559,177  
Art Unit: 1638

Page 15

If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

- (a) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;
- (b) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent;
- (c) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the enforceable life of the patent, whichever is longer;
- (d) a test of the viability of the biological material at the time of deposit (see 37 CFR 1.807); and,
- (e) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CFR 1.809(d) should be added to the specification. See 37 CFR 1.801 - 1.809 [MPEP 2401-2411.05] for additional explanation of these requirements.

### ***Claim Rejections - 35 USC § 102***

12. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:



Application/Control Number: 13/559,177  
Art Unit: 1638

Page 16

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

13. Claims 11 is rejected under 35 U.S.C. 102(b) as being anticipated by Strop et al (US Patent No. 4,808,426, issued February 28, 1989).

Claims 11 is drawn to a composition derived from the soybean plant of claim 8, wherein the composition is a commodity product such as soy oil. Claim 11 is found indefinite because the term “derived” is unclear. For the purpose of the examination, the claim is given the broadest reasonable interpretation as a product-by-process claim which are not limited by any process steps implied in the term “derived” but are only limited by the claimed structure, which in the instant case is a commodity product (MPEP 2113). The claimed commodity product, such as soy oil, would be indistinguishable from the oil obtained from another soybean plant.

Strop et al teach making soybean oil from soybean seed (Strop et al, claim 12). The oil of Strop et al will read on the soybean oil, of the instant claim 11. Strop et al thus anticipates the limitations of the instant claim 11.

14. Claims 8 and 9 are rejected under 35 U.S.C. 102(b) as being anticipated by Walker et al (J. Econ. Entomol. (2000) 93:613-622).

Applicant claims a soybean plant, or part thereof, or seed resistant to soybean looker and comprising a complement of DNA regions of SEQ ID NO: 1 and 2. Claims 8 and 9 were found indefinite. Because the claims do not specify the length of the complement, the complement would read on any dinucleotide, which would inherently

Application/Control Number: 13/559,177

Page 17

Art Unit: 1638

be present in the genome of any plant. Consequently, the claims would read on any soybean plants resistant to soybean looper.

Walker et al disclose a line of soybean plants transformed with Cry1Ac protein and resistant to soybean looper (Walker et al, pg. 614, left col., second full paragraph). Walker et al disclose the seeds from said line (Walker et al, pg. 614, left col., last paragraph). As a result, Walker et al anticipate the limitations of the instant claims 8 and 9.

### ***Claim Rejections - 35 USC § 103***

15. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

16. Claim 9 is rejected under 35 U.S.C. 103(a) as being unpatentable over Strop et al (US Patent No. 4,808,426, issued February 28, 1989) in view of Spencer et al (Plant Mol. Biol. (1992) 18:201-210).

Claim 9 is directed to the seed of the soybean plant of claim 8. Because the claims do not required that the seed be grown from the deposited seeds and comprise the event, the term “seeds” is given the broadest reasonable interpretation as encompassing the seeds obtained from the plant of any filial generation of the plants grown from the deposited seeds.

Application/Control Number: 13/559,177  
Art Unit: 1638

Page 18

Strop et al teach soybean seeds used for producing soybean oil and not comprising the instantly claimed event (Strop et al, claims 1, 12, 14 for example)

Spencer et al teach that plant transgenes, including transformation events, are inherited in a Mendelian fashion and thus can segregate in the progeny) (Spencer et al, Abstract, pg. 202, top of left col., pg. 209, left col.)

At the time the invention was made, at least some of the seed of a plant grown from the seeds of ATCC Seed Deposit PTA-12006 will not comprise the event (in view of the teachings of Spencer et al) and thus will be obvious variants of the seeds of Strop et al (or any wild-type soybean seeds), because the latter do not comprise the claimed event.

### ***Double Patenting***

17. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory obviousness-type double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir.

Application/Control Number: 13/559,177

Page 19

Art Unit: 1638

1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); and *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on a nonstatutory double patenting ground provided the conflicting application or patent either is shown to be commonly owned with this application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement.

Effective January 1, 1994, a registered attorney or agent of record may sign a terminal disclaimer. A terminal disclaimer signed by the assignee must fully comply with 37 CFR 3.73(b).

18. Claim 6 is are provisionally rejected on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 1 of copending Application No. 13/558,982 (U.S. Patent Application Publication 2013/0065230). Although the conflicting claims are not identical, they are not patentably distinct from each other. Claim 6 of the instant application is drawn to an isolated DNA sequence comprising one or more sequences selected from the group consisting of the regions of SEQ ID NO: 1 and 2 which regions encompass nucleotides 1200-1600 of SEQ ID NO: 1 and nucleotides 3-303 of SEQ ID NO: 2. These sequences comprise the portions of the flanking and the junction regions of the claimed soybean event 9582.814.19.1 (see Brief Description of the Sequences on pg. 7 of the instant specification). Claim 1 of the co-pending Application are drawn to a method of detecting soybean event pDAB9582.814.19.1,

Application/Control Number: 13/559,177

Page 20

Art Unit: 1638

said method comprising contacting a sample with primers of at least 10 bp that recognize a flanking sequence within base pairs 1-1400 and 1401-1836 of SEQ ID NO: 1 (of co-pending Application) and within base pairs 1-152 and 153-1550 of SEQ ID NO: 2 (of co-pending Application). The nucleotide sequences of the instant SEQ ID NO: 1 and 2 show 100% sequence identity to SEQ ID NO: 1 and 2 of the instant claims. The event of the co-pending Application also appears to be identical to the instantly claimed event. Although not identical, the claims not patentably distinct from each other, because the instantly claimed isolated DNA sequences are rendered obvious over the method claimed in the co-pending Application, given that the method comprise using identical DNA sequences.

This is a provisional obviousness-type double patenting rejection because the conflicting claims have not in fact been patented.

### ***Conclusion***

19. No claims are allowed.

20. Claims 1-8, 10, and 12 are free of prior art which neither teaches nor renders obvious a soybean plant comprising the instant SEQ ID NO: 1 and 2; or the soybean plant of event 9582.814.19.1, the representative seed of which was deposited with the ATCC under Accession No. 12006.

21. Any inquiry concerning this communication or earlier communications from the examiner should be directed to MYKOLA KOVALENKO whose telephone number is

Application/Control Number: 13/559,177

Page 21

Art Unit: 1638

(571) 272-6921. The examiner can normally be reached on Monday-Friday 8:30 am - 5 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisors, ANNE MARIE GRUNBERG and ZHOU (JOE) SHUBO can be reached at (571) 272-0975 and (571) 272-0724. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Mykola Kovalenko/  
Examiner, Art Unit 1638

/CATHY KINGDON WORLEY/  
Primary Examiner, Art Unit 1638



# **Exhibit S**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Attorney Ref. : DAS-P0292-01-US  
Applicants : Nathan Bard *et al.*  
Serial No. : 13/559,177  
Conf. No. : 6091  
Filed : July 26, 2012  
Title : INSECT RESISTANT AND HERBICIDE TOLERANT  
SOYBEAN EVENT 9582.814.19.1  
  
Art Unit : 1638  
Examiner : Kovalenko, Mykola V

**RESPONSE**

An Information Disclosure Statement accompanies this Response

This is a Response to the office action dated July 10, 2013. Reconsideration is respectfully requested.

AMENDMENTS TO THE SPECIFICATION begin on page 2 of this Response

AMENDMENTS TO THE CLAIMS begin on page 3 of this Response.

REMARKS begin on page 5 of this Response.

Application Serial No. 13/559,177  
Attorney Ref. DAS-P0292-01-US

IN THE SPECIFICATION

Please replace the first paragraph of page 1 with the following:

“This application claims ~~priority~~ the benefits of U.S. Provisional Application No. 61/511,664, filed July 26, 2011, and U.S. Provisional Application No. 61/521,798, filed August 10, 2011, both of which are herein incorporated by reference in their entireties.”

Please replace the abstract with the following:

“Soybean event 9582.814.19.1, wherein the event comprises genes encoding Cry1F, Cry1Ac (synpro), and PAT, affording insect resistance and herbicide tolerance to soybean crops containing the event, and enabling methods for crop protection and protection of stored products.”

No new matter is added by this amendment.

Application Serial No. 13/559,177  
Attorney Ref. DAS-P0292-01-US

**IN THE CLAIMS**

1. (currently amended) A method of controlling insects, said method comprising that comprises exposing insects to insect resistant soybean plants, said soybean plants comprising a DNA sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1, bp 1350-1450 of SEQ ID NO:1, bp 1300-1500 of SEQ ID NO:1, bp 1200-1600 of SEQ ID NO:1, bp 137-168 of SEQ ID NO:2, bp 103-203 of SEQ ID NO:2, and bp 3-303 of SEQ ID NO:2 SEQ ID NO:14, said sequence being diagnostic for the presence of soybean event 9582.814.19.1, to thereby control the insects.
2. (original) The method of claim 1 wherein said insects are *Pseudoplusia includens* (soybean looper).
3. (original) The method of claim 1 wherein said insects are *Anticarsia gemmatilis* (velvet bean caterpillar).
4. (original) The method of claim 1 wherein said insects are *Spodoptera frugiperda* (fall armyworm).
5. (currently amended) A method of controlling weeds in a soybean crop, that comprises said method comprising applying glufosinate herbicide to the soybean crop, said soybean crop comprising soybean plants comprising a DNA sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1, bp 1350-1450 of SEQ ID NO:1, bp 1300-1500 of SEQ ID NO:1, bp 1200-1600 of SEQ ID NO:1, bp 137-168 of SEQ ID NO:2, bp 103-203 of SEQ ID NO:2, and bp 3-303 of SEQ ID NO:2 SEQ ID NO:14, said sequence being diagnostic for the presence of soybean event 9582.814.19.1.
6. (cancelled)
7. (currently amended) A method of breeding a soybean plant, said method comprising: crossing a first plant with a second soybean plant to produce a third soybean plant, said first plant comprising DNA comprising one or more sequences selected from the group consisting of 1385-1415 of SEQ ID NO:1, bp 1350-1450 of SEQ ID NO:1, bp 1300-1500 of SEQ ID NO:1, bp 1200-1600 of SEQ ID NO:1, bp 137-168 of SEQ ID NO:2, bp 103-203 of SEQ ID

Application Serial No. 13/559,177  
Attorney Ref. DAS-P0292-01-US

~~NO:2, and bp 3-303 of SEQ ID NO:2, and complements thereof SEQ ID NO:14; and assaying said third soybean plant for the presence of DNA comprising one or more sequences selected from the group consisting of bp 1385-1415 of SEQ ID NO:1, bp 1350-1450 of SEQ ID NO:1, bp 1300-1500 of SEQ ID NO:1, bp 1200-1600 of SEQ ID NO:1, bp 137-168 of SEQ ID NO:2, bp 103-203 of SEQ ID NO:2, by 3-303 of SEQ ID NO:2, and complements thereof SEQ ID NO:14.~~

8. (currently amended) A soybean plant, wherein representative seed of said soybean plant has been deposited with the American Type Culture Collection under Accession No. PTA-12006 or part thereof, that is resistant to Pseudoplusia includens (soybean looper) and comprises DNA having at least one nucleotide sequence selected from the group consisting of bp 1385-1415 of SEQ ID NO:1, bp 1350-1450 of SEQ ID NO:1, bp 1300-1500 of SEQ ID NO:1, bp 1200-1600 of SEQ ID NO:1, bp 137-168 of SEQ ID NO:2, bp 103-203 of SEQ ID NO:2, bp 3-303 of SEQ ID NO:2, and complements thereof.

9. (currently amended) A seed or a part of the plant of claim 8, wherein said seed or part comprises SEQ ID NO:14.

10. (currently amended) ~~The A~~ soybean plant, or part thereof, of claim 8 comprising a DNA sequence having at least 95% sequence identity ~~with to~~ SEQ ID NO:14, ~~wherein said soybean plant has been deposited with American Type Culture Collection under Accession No. PTA-12006.~~

11. (cancelled)

12. (cancelled)

Application Serial No. 13/559,177  
Attorney Ref. DAS-P0292-01-US

### **REMARKS**

#### **Claims Status:**

Claims 1-12 were the subject of the office action dated July 10, 2013. The applicants cancel claims 6, 11, and 12, and amend claims 1, 5, 7, 9, and 10 without prejudice. Thus, claims 1-5, and 7-10 are for reconsideration.

#### **Specification:**

The Specification is amended according to the Examiner's suggestions. No new matter is introduced.

#### **Claim Objections:**

Claims 1, 5, 7 and 10 are amended according to the Examiner's suggestions. Thus, these objections are moot. The withdrawal of these objections is requested.

#### **Claim Rejections under 35 USC §112 (b):**

These rejections should be rendered moot. Specific reference to complements is removed from claim 7, for example. Claim 8 now refers to the seed deposit. Claim 10 now refers only to the event sequence SEQ ID NO:14 (as reference to the seed deposit is moved to claim 8.) Claims 11 and 12 are canceled without prejudice. Thus, the withdrawal of the 112(b) rejection is respectfully requested.

#### **Claim Rejections under 35 USC §112 (a):**

Claims 1-5 and 7-12 stand rejected under 35 USC §112 (a) for lack of adequate description. Aside from claim 8, the only sequence the claims now refer to is the event sequence SEQ ID NO:14 (5' genomic flanking, insert, and 3' genomic flanking sequence). It is also known in the art that there can be sequence errors and variation in genomic flanking and insertion sequences that randomly occur during transformation and insertion, for example.



Application Serial No. 13/559,177  
Attorney Ref. DAS-P0292-01-US

Claims 1-5 and 7-12 stand rejected under 35 USC §112 (a) for lack of enablement. Aside from claim 8, the only sequence the claims now refer to is the event sequence SEQ ID NO:14 (5' genomic flanking, insert, and 3' genomic flanking sequence). In addition, a Budapest Treaty deposit statement and the corresponding Deposit Receipt are submitted with this response.

Thus, the withdrawal of the rejections under 35 USC §112 (a) is respectfully requested.

Claim rejections under 35 USC §102 (b):

Claim 11 is canceled, which renders the rejection to claim 11 under 102 (b) moot. Claim 8 now refers to the seed deposit. Thus, the withdrawal of the 102 (b) rejection is respectfully requested.

Claim rejections under 35 USC §103 (a):

Claim 9 stands rejected under 35 USC §103 (a) as obvious over Strop et al (US Patent No. 4,808,426) in view of Spencer et al. Claim 9 now refers to SEQ ID NO:14 (to clarify that the claimed seed comprises the event). Thus, the withdrawal of the 103 (a) rejection is respectfully requested.

Double Patenting:

Claim 6 was provisionally rejected for non-statutory obviousness double patenting. Claim 6 is canceled without prejudice, so this rejection should be rendered moot.

Conclusion:

In light of foregoing, the application is believed to be in condition of allowance, and such action is respectfully requested.

Any fees due in connection with this response are hereby authorized to be charged to Deposit Account No. 02-0390 but not to include any issue fees.

Application Serial No. 13/559,177  
Attorney Ref. DAS-P0292-01-US

Respectfully submitted,



Jay M. Sanders, Reg. No. 39,355  
Faegre Baker Daniels LLP  
300 N. Meridian Street, Suite 2700  
Indianapolis, Indiana 46204  
Telephone: (317) 237-1245  
Fax: (317) 237-1000

Attachments:

Budapest Treaty Seed Deposit Statement  
IDS

Submitted Oct. 10, 2013

# **Exhibit T**



## UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office  
Address: COMMISSIONER FOR PATENTS  
P.O. Box 1450  
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## NOTICE OF ALLOWANCE AND FEE(S) DUE

83067 7590 11/05/2013  
Faegre Baker Daniels LLP- Dow AgroSciences  
300 North Meridian Street, Suite 2700  
Indianapolis, IN 46204

EXAMINER

KOVALENKO, MYKOLA V

ART UNIT

PAPER NUMBER

1638

DATE MAILED: 11/05/2013

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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13/559,177

07/26/2012

Nathan Bard

DAS-P0292-01-US

6091

TITLE OF INVENTION: INSECT RESISTANT AND HERBICIDE TOLERANT SOYBEAN EVENT 9582.814.19.1

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1780	\$300	\$0	\$2080	02/05/2014

**THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.**

**THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.**

**HOW TO REPLY TO THIS NOTICE:**

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

**IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.**

PART B - FEE(S) TRANSMITTAL

# 7743

Complete and send this form, together with applicable fee(s), to: **Mail** **Mail Stop ISSUE FEE**  
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INSTRUCTIONS: This form should be used for transmitting the ISSUE FEE and PUBLICATION FEE (if required). Blocks 1 through 5 should be completed where appropriate. All further correspondence including the Patent, advance orders and notification of maintenance fees will be mailed to the current correspondence address as indicated unless corrected below or directed otherwise in Block 1, by (a) specifying a new correspondence address; and/or (b) indicating a separate "FEE ADDRESS" for maintenance fee notifications.

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83067 7590 11/05/2013  
**Faegre Baker Daniels LLP- Dow AgroSciences**  
**300 North Meridian Street, Suite 2700**  
**Indianapolis, IN 46204**

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I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/559,177	07/26/2012	Nathan Bard	DAS-P0292-01-US	6091

TITLE OF INVENTION: INSECT RESISTANT AND HERBICIDE TOLERANT SOYBEAN EVENT 9582.814.19.1

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$1780	\$300	\$0	\$2080	02/05/2014

EXAMINER	ART UNIT	CLASS-SUBCLASS
KOVALENKO, MYKOLA V	1638	800-265000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) the names of up to 3 registered patent attorneys or agents OR, alternatively,

1 \_\_\_\_\_

(2) the name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

2 \_\_\_\_\_

3 \_\_\_\_\_

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent) : ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

☐ Issue Fee

☐ Publication Fee (No small entity discount permitted)

☐ Advance Order - # of Copies \_\_\_\_\_

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)

☐ A check is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The Director is hereby authorized to charge the required fee(s), any deficiency, or credit any overpayment, to Deposit Account Number \_\_\_\_\_ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)☐ Applicant certifying micro entity status. See 37 CFR 1.29

NOTE: Absent a valid certification of Micro Entity Status (see form PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

☐ Applicant asserting small entity status. See 37 CFR 1.27

NOTE: If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

☐ Applicant changing to regular undiscounted fee status.

NOTE: Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: The Issue Fee and Publication Fee (if required) will not be accepted from anyone other than the applicant; a registered attorney or agent; or the assignee or other party in interest as shown by the records of the United States Patent and Trademark Office.

Authorized Signature \_\_\_\_\_

Date \_\_\_\_\_

Typed or printed name \_\_\_\_\_

Registration No. \_\_\_\_\_

This collection of information is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450.

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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
13/559,177	07/26/2012	Nathan Bard	DAS-P0292-01-US	6091

83067 7590 11/05/2013  
 Faegre Baker Daniels LLP- Dow AgroSciences  
 300 North Meridian Street, Suite 2700  
 Indianapolis, IN 46204

EXAMINER
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KOVALENKO, MYKOLA V

ART UNIT	PAPER NUMBER
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1638

DATE MAILED: 11/05/2013

### Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)

(application filed on or after May 29, 2000)

The Patent Term Adjustment to date is 0 day(s). If the issue fee is paid on the date that is three months after the mailing date of this notice and the patent issues on the Tuesday before the date that is 28 weeks (six and a half months) after the mailing date of this notice, the Patent Term Adjustment will be 0 day(s).

If a Continued Prosecution Application (CPA) was filed in the above-identified application, the filing date that determines Patent Term Adjustment is the filing date of the most recent CPA.

Applicant will be able to obtain more detailed information by accessing the Patent Application Information Retrieval (PAIR) WEB site (<http://pair.uspto.gov>).

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

## Privacy Act Statement

**The Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

**Notices of Allowance and Fee(s) Due mailed between October 1, 2013 and December 31, 2013**

(Addendum to PTOL-85)

If the “Notice of Allowance and Fee(s) Due” has a mailing date on or after October 1, 2013 and before January 1, 2014, the following information is applicable to this application.

If the issue fee is being timely paid on or after January 1, 2014, the amount due is the issue fee and publication fee in effect January 1, 2014. On January 1, 2014, the issue fees set forth in 37 CFR 1.18 decrease significantly and the publication fee set forth in 37 CFR 1.18(d)(1) decreases to \$0.

If an issue fee or publication fee has been previously paid in this application, applicant is not entitled to a refund of the difference between the amount paid and the amount in effect on January 1, 2014.

<b>Notice of Allowability</b>	<b>Application No.</b> 13/559,177	<b>Applicant(s)</b> BARD ET AL.	
	<b>Examiner</b> MYKOLA KOVALENKO	<b>Art Unit</b> 1638	<b>AIA (First Inventor to File) Status</b> No

**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--**

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to Amendments and Remarks filed on October 10, 2013.  
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on \_\_\_\_\_.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on \_\_\_\_\_; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are 1-5, 7-10. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see [http://www.uspto.gov/patents/init\\_events/oph/index.jsp](http://www.uspto.gov/patents/init_events/oph/index.jsp) or send an inquiry to [PPHfeedback@uspto.gov](mailto:PPHfeedback@uspto.gov).
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a) ☐ All    b) ☐ Some    \*c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

\* Certified copies not received: \_\_\_\_\_.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.  
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS ( as "replacement sheets") must be submitted.  
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date \_\_\_\_\_.

**Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

<ol style="list-style-type: none"> <li>1. <input type="checkbox"/> Notice of References Cited (PTO-892)</li> <li>2. <input checked="" type="checkbox"/> Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____</li> <li>3. <input type="checkbox"/> Examiner's Comment Regarding Requirement for Deposit of Biological Material</li> <li>4. <input checked="" type="checkbox"/> Interview Summary (PTO-413), Paper No./Mail Date <u>20131024</u>.</li> </ol>	<ol style="list-style-type: none"> <li>5. <input checked="" type="checkbox"/> Examiner's Amendment/Comment</li> <li>6. <input type="checkbox"/> Examiner's Statement of Reasons for Allowance</li> <li>7. <input type="checkbox"/> Other _____.</li> </ol>
--	--

/MYKOLA KOVALENKO/ Examiner, Art Unit 1638	/Cathy Kingdon Worley/ Primary Examiner, Art Unit 1638
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Application/Control Number: 13/559,177  
Art Unit: 1638

Page 2

### **EXAMINER'S COMMENT**

1. The present application is being examined under the pre-AIA first to invent provisions.
2. The amendments submitted on October 10, 2013, have been entered. Claims 6, 11, and 12 have been canceled. Claims 1-5 and 7-10 are pending.

### ***Examiner's Amendment***

3. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Jay M. Sanders, Applicant's attorney, on October 24, 2013.

The claims have been amended as follows:

Claim 1. (currently amended) A method of controlling insects, said method comprising exposing insects to insect resistant soybean plants, said soybean plants comprising SEQ ID NO: 14, wherein said insects are selected from the group consisting of: Pseudoplusia includens (soybean looper), Anticarsia gemmatalis (velvet bean caterpillar), and Spodoptera frugiperda (fall armyworm).

Claim 10. (currently amended) A soybean plant, or part thereof, comprising ~~the DNA sequence having at least 95% sequence identity to~~ of SEQ ID NO: 14.

Application/Control Number: 13/559,177  
Art Unit: 1638

Page 3

***Allowable Subject Matter***

4. Claims 1-5 and 7-10 are allowed and are renumbered as claims 1-9, respectively.

***Contact Information***

5. Any inquiry concerning this communication or earlier communications from the examiner should be directed to MYKOLA KOVALENKO whose telephone number is (571) 272-6921. The examiner can normally be reached on Monday-Friday 8:30 am - 5 pm EST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisors, ANNE MARIE GRUNBERG and ZHOU (JOE) SHUBO can be reached at (571) 272-0975 and (571) 272-0724. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.



Application/Control Number: 13/559,177  
Art Unit: 1638

Page 4

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/Mykola Kovalenko/  
Examiner, Art Unit 1638

/CATHY KINGDON WORLEY/  
Primary Examiner, Art Unit 1638

# **Exhibit U**



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/091,896	11/03/2008	Terry R. Wright	DAS-PO129-US-02	6428
83067	7590	07/14/2011	EXAMINER	
Baker & Daniels LLP- Dow AgroSciences 300 North Meridian Street, Suite 2700 Indianapolis, IN 46204			KRUSE, DAVID H	
			ART UNIT	PAPER NUMBER
			1638	
			NOTIFICATION DATE	DELIVERY MODE
			07/14/2011	ELECTRONIC

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

inteas@bakerd.com  
cynthia.payson@bakerd.com

# 7754

<b>Office Action Summary</b>	<b>Application No.</b>	<b>Applicant(s)</b>
	12/091,896	WRIGHT ET AL.
	<b>Examiner</b>	<b>Art Unit</b>
	DAVID H. KRUSE	1638

-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --

### Period for Reply

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

### Status

- 1) ☐ Responsive to communication(s) filed on \_\_\_\_.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

### Disposition of Claims

- 4) ☒ Claim(s) 89-103 is/are pending in the application.
- 4a) Of the above claim(s) \_\_\_\_ is/are withdrawn from consideration.
- 5) ☐ Claim(s) \_\_\_\_ is/are allowed.
- 6) ☒ Claim(s) 89-103 is/are rejected.
- 7) ☐ Claim(s) \_\_\_\_ is/are objected to.
- 8) ☐ Claim(s) \_\_\_\_ are subject to restriction and/or election requirement.

### Application Papers

- 9) ☒ The specification is objected to by the Examiner.
- 10) ☒ The drawing(s) filed on 28 April 2008 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 11) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

### Priority under 35 U.S.C. § 119

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some \* c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

### Attachment(s)

- |  |  |
|--|--|
| <p>1) <input checked="" type="checkbox"/> Notice of References Cited (PTO-892)</p> <p>2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948)</p> <p>3) <input checked="" type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)<br/>Paper No(s)/Mail Date <u>7/20/2010</u>.</p> | <p>4) <input type="checkbox"/> Interview Summary (PTO-413)<br/>Paper No(s)/Mail Date. ____.</p> <p>5) <input type="checkbox"/> Notice of Informal Patent Application</p> <p>6) <input type="checkbox"/> Other: ____.</p> |
|--|--|

Application/Control Number: 12/091,896  
Art Unit: 1638

Page 2

## **DETAILED ACTION**

### ***Information Disclosure Statement***

1. The listing of references on pages 135-142 of the specification is not a proper information disclosure statement. 37 CFR § 1.98(b) requires a list of all patents, publications, or other information submitted for consideration by the Office, and MPEP § 609.04(a) states, "the list may not be incorporated into the specification but must be submitted in a separate paper." Therefore, unless the references have been cited by the examiner on form PTO-892, they have not been considered.

### ***Specification***

2. The disclosure is objected to because of the following informalities:

On page 106 there is a Table that lacks a table number, subsequent Tables would need to be renumbered and the specification amended to reflect the renumbering of Tables.

At pages 108-109, paragraph 00361, there appear to be nucleotide sequences not in the Sequence Listing. This paragraph refers to sequence numbers found in a PCT application. If the recited nucleotide sequences are not found in the instant Sequence Listing, Applicants are required to place the instant application in compliance with the Sequence Rules. Failure to correct this issue will be held as non-responsive to this Office action.

Appropriate correction is required.

Application/Control Number: 12/091,896  
Art Unit: 1638

Page 3

***Claim Objections***

3. The claims are objected to because of the following informalities: Specifically in the Claims amendment filed on 28 April 2008, the listing of claims should indicate -- 1-88 (cancelled) --; in addition none of the newly presented claims have a status identifier. Any response to this Office action must present the claims in compliance with 37 CFR § 1.121(c). Failure to correct this issue will be held as non-responsive to this Office action. Appropriate correction is required.

***Claim Rejections - 35 USC § 101***

4. 35 U.S.C. § 101 reads as follows:

Whoever invents or discovers any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof, may obtain a patent therefor, subject to the conditions and requirements of this title.

5. Claim 103 is rejected under 35 U.S.C. § 101 because the claimed invention is directed to non-statutory subject matter. The claim reads on a product of nature, the recitation of structural and functional characteristics does not specifically denote the hand of man in the instantly claimed invention. A DNA sequence is not patentable because a sequence is merely descriptive information about a molecule. Amending the claim to read -- An isolated polynucleotide -- would obviate this rejection.

***Claim Rejections - 35 USC § 112***

6. The following is a quotation of the first paragraph of 35 U.S.C. § 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

7. Claims 89-103 are rejected under 35 U.S.C. § 112, first paragraph, as failing to comply with the written description requirement. The claim(s) contains subject matter



Application/Control Number: 12/091,896  
Art Unit: 1638

Page 4

which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention.

Applicants claim a polynucleotide encoding an aryloxyalkanoate dioxygenase enzyme that hybridizes under stringent conditions comprising 0.2x SSPE at 65°C to the full complement of instant SEQ ID NO: 1, 3 or 5. Applicants claim a plant cell comprising said polynucleotide. Applicants claim a plant cell wherein the enzyme has at least 85% sequence identity with SEQ ID NO: 2 or SEQ ID NO: 4.

Applicants describe isolated polynucleotides that encode an aryloxyalkanoate dioxygenase enzyme having the amino acid sequence of SEQ ID NO: 2 or SEQ ID NO: 4 (which are 99.7% identical to each other). Applicants describe three polynucleotides that encode instant SEQ ID NO: 2; SEQ ID NO: 1 being a native coding sequence and SEQ ID NO: 3 and 5 being codon optimized coding sequences. SEQ ID NO: 1 is 74.5% identical to SEQ ID NO: 5 and 67.9% identical to SEQ ID NO: 3.

Applicants do not describe the broad genus of polynucleotides encoding an aryloxyalkanoate dioxygenase enzyme that hybridizes under stringent conditions comprising 0.2x SSPE at 65°C to the full complement of instant SEQ ID NO: 1, 3 or 5, or that encode said enzyme wherein the enzyme has at least 85% sequence identity with SEQ ID NO: 2 or SEQ ID NO: 4.

Hence, it is unclear that Applicants were in possession of the invention as broadly claimed. See *Ex parte Kubin*, 83 USPQ2d 1410 (Bd. Pat. App. & Int. 2007) at pg 1417:

Application/Control Number: 12/091,896  
Art Unit: 1638

Page 5

[Appellants] ... have not described what domains of those sequences are correlated with the required binding to CD48, and thus have not described which of NAIL's amino acids can be varied and still maintain binding. Thus, under Lilly and its progeny, their Specification would not have shown possession of a sufficient number of sequences falling within their potentially large genus to establish possession of their claimed genus. Cf. *Enzo*, 323 F.3d at 964, 63 USPQ2d at 1612 ("if the functional characteristic of ... binding to [CD48] were coupled with a disclosed correlation between that function and a structure that is sufficiently known or disclosed," the written description requirement may be met).

Without a correlation between structure and function, the claim does little more than define the claimed invention by function. That is not sufficient to satisfy the written description requirement. See *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 ("definition by function ... does not suffice to define the genus because it is only an indication of what the gene does, rather than what it is").

With respect to Appellants' reliance on hypothetical Example 14 in the Office's Synopsis, "[c]ompliance with the written description requirement is essentially a fact-based inquiry that will 'necessarily vary depending on the nature of the invention claimed.'" *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991) (quoting *In re DiLeone*, 436 F.2d 1404, 1405, 168 USPQ 592, 593 (CCPA 1971)), quoted with approval in *Enzo*, 323 F.3d at 963, 63 USPQ2d at 1612. While the Written Description Guidelines and the hypothetical examples in the Office's *Synopsis* can be helpful in understanding how to apply the relevant law (as it existed in 2001 when the Guidelines were adopted), they do not create a rigid test.

### ***Claim Rejections - 35 USC § 102***

8. The following is a quotation of the appropriate paragraphs of 35 U.S.C. § 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

9. Claim 103 is rejected under 35 U.S.C. § 102(b) as being anticipated by Schleinitz *et al* (September 2004, *Applied and Environmental Microbiology* 70(9): 5357-5365).

Schleinitz *et al* disclose an isolated polynucleotide comprising instant SEQ ID NO: 1 at GenBank accession number AY327575 (page 5359, right column, 5<sup>th</sup> paragraph). The encoded protein (see FIG 2 on page 5360) would inherently have

Application/Control Number: 12/091,896

Page 6

Art Unit: 1638

aryloxyalkanoate dioxygenase activity. Hence, the claim limitation had been previously disclosed.

10. Claims 89-94 and 103 are rejected under 35 U.S.C. 102(b) as being anticipated by Kaphammer (U.S. Patent 5,608,147) taken with the evidence of Fourgoux-Nicol *et al* (1999, Plant Molecular Biology 40: 857-872).

Kaphammer discloses a plant cell comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, resistance to 2,4-dichlorophenoxyacetate (2,4-D) at claim 11, and a plant regenerated therefrom at claim 15. Kaphammer discloses an isolated polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity at SEQ ID NO: 1.

Fourgoux-Nicol *et al* (1999, Plant Molecular Biology 40 :857-872) teach the isolation of a 674bp fragment using a 497bp probe incorporating stringent hybridization conditions comprising three consecutive 30 minute rinses in 2X, 1X and 0.1X SSC with 0.1% SDS at 65°C (page 859, left column, 2<sup>nd</sup> paragraph). Fourgoux-Nicol *et al* also teach that the probe and isolated DNA fragment exhibited a number of sequence differences comprising a 99bp insertion within the probe and a single nucleotide gap, while the DNA fragment contained 2 single nucleotide gaps and together the fragments contained 27 nucleotide mismatches. Taking into account the insertions, gaps and mismatches, the longest stretch of contiguous nucleotides to which the probe could hybridize consisted of 93bp of DNA (page 862, Figure 2). In the present example, the isolated fragment exhibits less than 50% sequence identity with the probe.

Application/Control Number: 12/091,896  
Art Unit: 1638

Page 7

Without evidence to the contrary, the polynucleotide disclosed by Kaphammer would hybridize to instant SEQ ID NO: 1 or 3, for example, under stringent conditions comprising 0.2X SSPE at 65°C.

Kaphammer discloses transforming a soybean cell at Example 11, columns 21-22. Kaphammer discloses a method of controlling weeds by applying 2,4-D herbicide to a crop field at column 2, lines 26-28. Hence, Kaphammer had previously disclosed the claim limitations.

***Claim Rejections - 35 USC § 103***

11. The following is a quotation of 35 U.S.C. 103(a) which forms the basis for all obviousness rejections set forth in this Office action:

(a) A patent may not be obtained though the invention is not identically disclosed or described as set forth in section 102 of this title, if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to a person having ordinary skill in the art to which said subject matter pertains. Patentability shall not be negated by the manner in which the invention was made.

12. Claims 89-102 are rejected under 35 U.S.C. 103(a) as being unpatentable over Kaphammer (U.S. Patent 5,608,147) in view of Schleinitz *et al* (September 2004, Applied and Environmental Microbiology 70(9): 5357-5365) and Pallett *et al* (U.S. Patent 7,205,561 B1, § 371(c)(1) date of 15 June 1998).

Kaphammer teaches a plant cell comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, resistance to 2,4-dichlorophenoxyacetate (2,4-D) at claim 11, and a plant regenerated therefrom at claim 15. Kaphammer teaches an isolated polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity at SEQ ID NO: 1. Kaphammer teaches transforming a soybean cell at Example 11, columns 21-22. Kaphammer teaches a

Application/Control Number: 12/091,896

Page 8

Art Unit: 1638

method of controlling weeds by applying 2,4-D herbicide to a crop field at column 2, lines 26-28. Kaphammer teaches that a transgene the confers resistance to glyphosate can also be introduced into the plant cell (column 3, lines 11-6).

Kaphammer does not teach an isolated nucleic acid encoding instant SEQ ID NO: 2 or having the nucleotide sequence of instant SEQ ID NO: 1. Kaphammer does not teach introducing a polynucleotide that confers glufosinate resistance.

Schleinitz *et al* teach an isolated polynucleotide comprising instant SEQ ID NO: 1 at GenBank accession number AY327575 (page 5359, right column, 5<sup>th</sup> paragraph). The encoded protein (see FIG 2 on page 5360) would naturally have aryloxyalkanoate dioxygenase activity.

Pallett *et al* teach introducing polynucleotides to a transformed plant cell that renders said plant cell resistant to multiple herbicides including glyphosate (column 1, lines 62-64) and glufosinate (column 2, lines 27-29).

It would have been *prima face* obvious to one of ordinary skill in the art at the time of Applicants' invention to modify the teachings of Kaphammer to introduce a polynucleotide encoding instant SEQ ID NO: 2 as taught by Schleinitz *et al* in addition to a polynucleotide encoding resistance to glyphosate and glufosinate as suggested Pallett *et al*. Given the success of Pallett *et al*, and Kaphammer one of ordinary skill in the art would have had a reasonable expectation of success. Given dichlorprop was a known herbicide as taught by Schleinitz *et al* (page 5357, left column, 1<sup>st</sup> paragraph), one of ordinary skill in the art would have been motivated to introduce said polynucleotide encoding instant SEQ ID NO: 2 into a plant cell. Application of herbicide prior to

Application/Control Number: 12/091,896

Page 9

Art Unit: 1638

planting (instant claim 95) or as a pre-emergence application (instant claim 96) to control weeds would have been obvious to one of ordinary skill in the art at the time of Applicants' invention as such application would have been a design choice in the instant art.

### ***Conclusion***

13. No claims are allowed.

14. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571) 272-0799. The examiner can normally be reached on Monday to Friday from 8:00 a.m. to 4:30 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anne Marie Grunberg can be reached at (571) 272-0975. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/  
Primary Examiner, Art Unit 1638  
8 July 2011



# **Exhibit V**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Art Unit : 1616  
Applicants : Terry R. Wright, Justin M. Lira, Terence A. Walsh, Donald J. Merlo,  
Pon Samuel Jayakumar, Gaofeng Lin.  
Attorney ref. : DAS-P0129-US-02  
Serial No. : 12/091,896  
Conf. No. : 6428  
Filed : April 28, 2008.  
For : Novel Herbicide Resistance Genes

RESPONSE

In response to the office action dated July 14, 2011, reconsideration is respectfully requested. Please amend the subject application as follows.

In the Specification:

Between paragraphs 37 and 38, please insert the following:

**SEQ ID NO:16** provides the sequence of forward primer brjap 5'(speI).

**SEQ ID NO:17** provides the sequence of reverse primer br jap 3' (xhoI).

Please replace paragraph 361 (pages 108-109) as follows:

The strain of *Bradyrhizobium japonicum* containing the *AAD-2 (v1)* gene was obtained from Northern Regional Research Laboratory (NRRL, strain #B4450). The lyophilized strain was revived according to NRRL protocol and stored at -80° C in 20% glycerol for internal use as Dow Bacterial strain DB 663. From this freezer stock, a plate of Tryptic Soy Agar was then struck out with a loopful of cells for isolation, and incubated at 28° C for 3 days. A single colony was used to inoculate 100 ml of Tryptic Soy Broth in a 500 ml tri-baffled flask, which was incubated overnight at 28° C on a floor shaker at 150 rpm. From this, total DNA was isolated with the gram negative protocol of Qiagen's DNeasy kit (Qiagen cat. #69504). The following primers were designed to amplify the target gene from genomic DNA, Forward (SEQ ID NO:16): 5' ACT AGT AAC AAA GAA GGA GAT ATA CCA TGA CGA T 3' [(brjap 5'(speI) SEQ ID NO:14 of PCT/US2005/014737 (added Spe I restriction site and Ribosome Binding Site (RBS))] and Reverse (SEQ ID NO:17): 5' TTC TCG AGC TAT CAC TCC GCC GCC TGC TGC TGC 3' [(br jap 3' (xhoI) SEQ ID NO:15 of PCT/US2005/014737 (added a Xho I site)].

In the Claims

89 (currently amended). A plant cell comprising a polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4a ~~nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, wherein said stringent conditions comprise 0.2x SSPE at 65° C.~~

90 (previously presented). A plant comprising a plurality of cells of claim 89.

91 (previously presented). A method of controlling weeds in a crop field, said method comprising applying an aryloxyalkanoate herbicide to said crop field, said crop field comprising a plurality of plants, each said plant comprising a plurality of plant cells of claim 89, wherein expression of said polynucleotide renders said plant resistant or tolerant to said aryloxyalkanoate herbicide.

92 (previously presented). The method of claim 91 wherein said aryloxyalkanoate herbicide is 2,4-D.

93 (previously presented). The method of claim 91 wherein said plants are dicots.

94 (previously presented). The method of claim 93 wherein said dicots are soybean plants.

95 (previously presented). The method of claim 91 wherein said method further comprises applying said aryloxyalkanoate herbicide to said crop field prior to planting seeds in said field.

96 (previously presented). The method of claim 91 wherein said method further comprises applying said aryloxyalkanoate herbicide to said crop field after seeds are planted in said field but prior to emergence of said plants grown from said seeds.

97 (previously presented). The method of claim 91 wherein said method further comprises applying glyphosate to said crop field, wherein said plants further comprise a second polynucleotide wherein expression of said second polynucleotide renders said plants resistant or tolerant to glyphosate.

98 (previously presented). The method of claim 91 wherein said method further comprises applying glufosinate to said crop field, wherein said plants further comprise a second polynucleotide wherein expression of said second polynucleotide renders said plants resistant or tolerant to glufosinate.

99 (previously presented). The method of claim 91 wherein said method further comprises applying glyphosate and glufosinate to said crop field, wherein said plants further comprise a second polynucleotide wherein expression of said second polynucleotide renders said plants resistant or tolerant to glyphosate, and wherein said plants further comprise a third polynucleotide wherein expression of said third polynucleotide renders said plants resistant or tolerant to glufosinate.

100 (currently amended). The plant cell of claim 89 wherein said protein has at least 99.85% amino acid sequence identity with SEQ ID NO:2 or SEQ ID NO:4.

101 (previously presented). A seed comprising a plant cell of claim 89.

102 (previously presented). The plant of claim 90 wherein said plant further comprises an insect-resistance gene.

103 (currently amended). ~~A~~ An isolated polynucleotide that encodes a protein having aryloxyalkanoate dioxygenase activity, wherein said protein has at least 95% amino acid sequence identity with a sequence selected from the group consisting of SEQ ID NO:2 and SEQ ID NO:4 ~~a nucleic acid molecule that encodes said protein hybridizes under stringent conditions with the full complement of a sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, wherein said polynucleotide comprises a codon composition that is optimized for expression in a plant, and wherein said stringent conditions comprise 0.2x SSPE at 65° C.~~

Remarks

Claims 89-103 were the subject of the office action dated July 14, 2011. These claims are again presented for further consideration. Percent identity is now used in place of the hybridization language in claims 89 and 103. Claim 100 is amended accordingly.

The Table on page 106 was already identified in the specification as filed as Table 26 (top left corner of Table).

The two sequences from paragraph 361 (pages 108-109) are now added to the sequence listing and to the Brief Description of the Sequences, as indicated above. No new matter is added.

Claim 103 stands rejected under §101 as being directed to non-statutory subject matter. Although this claim specifies that the polynucleotide "comprises a codon composition that is optimized for expression in a plant" (and is therefore directed to non-naturally occurring subject matter), the term "isolated" is also now added to this claim as suggested by the examiner.

Claims 89-103 stand rejected under §112, first paragraph, as failing the written description requirement. As noted above, for purposes of further clarification, percent identity language is now used in claims 89 and 103 in place of the hybridization language. The examiner did take note of protein sequences SEQ ID NO: 2 and 4 (the latter of which is encoded by one of the optimized genes). Thus, the specification provides species for the claimed genus. Thus, this rejection should be rendered moot.

Claim 103 stands rejected as being anticipated by Schleinitz (September 2004, Applied and Environmental Microbiology 70(9): 5357-5365 / GenBank Accession No. AY327575). Schleinitz relates to bacterial genes. As noted above, claim 103 specified that the claimed polynucleotide "comprises a codon composition that is optimized for expression in a plant." Schleinitz does not teach the use of plant codons to increase expression of those genes. In light of the foregoing, the withdrawal of this rejection is respectfully requested.



Claims 89-94 and 103 stand rejected as being anticipated by U.S. Patent No. 5,608,147 (Kaphammer) "taken with the evidence of" Fourgoux-Nicol (1999, Plant Molecular Biology 40: 857-872). Kaphammer relates to *tfdA*, which shares only about 31% identity with SEQ ID NO:2 (AAD-12). In light of the percent identity ranges specified for purposes of further clarification in claims 89 and 103, this rejection should be moot.

Claims 89-102 stand rejected as being obvious over Kaphammer in view of Schleinitz and U.S. Patent No. 7,250,561 to Pallett. Kaphammer is distinguished above. Pallett is cited as teaching plant transformation in general. The examiner states that it would have thus been obvious to introduce the gene of Schleinitz into a plant with a reasonable expectation of success. The applicants respectfully traverse this rejection.

Example 1 of the subject specification states as follows (emphasis added):

As a way to identify genes which possess herbicide degrading activities *in planta*, it is possible to mine current public databases such as NCBI (National Center for Biotechnology Information). To begin the process, it is necessary to have a functional gene sequence already identified that encodes a protein with the desired characteristics (*i.e.*,  $\alpha$ -ketoglutarate dioxygenase activity). This protein sequence is then used as the input for the BLAST (Basic Local Alignment Search Tool) (Altschul *et al.*, 1997) algorithm to compare against available NCBI protein sequences deposited. **Using default settings, this search returns upwards of 100 homologous protein sequences at varying levels. These range from highly identical (85-98%) to very low identity (23-32%) at the amino acid level.** Traditionally only sequences with high homology would be expected to retain similar properties to the input sequence. In this case, only sequences with  $\leq 50\%$  homology were chosen. As exemplified herein, cloning and recombinantly expressing homologues with as little as 31% amino acid conservation (relative to *tfdA* from *Ralstonia eutropha*) can be used to impart commercial levels of resistance not only to the intended herbicide, but also to substrates never previously tested with these enzymes.

A single gene (*sdpA*) was identified from the NCBI database (*see the ncbi.nlm.nih.gov website; accession #AF516752*) as a homologue **with only 31% amino acid identity to *tfdA***. Percent identity was determined by first translating both the *sdpA* and *tfdA* DNA sequences deposited in the database to proteins, then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment.

Thus, the gene/protein of SEQ ID NO:2 was selected from many possible options (over 100).

In addition, Example 14 (entitled "Further Evidence of Surprising Results: AAD-12 versus AAD-2") of the subject specification states (with emphasis added):

Another gene was identified from the NCBI database (see the [ncbi.nlm.nih.gov](http://ncbi.nlm.nih.gov) website; accession #AP005940) as a homologue with only 44% amino acid identity to *tfdA*. This gene is referred to herein as *AAD-2 (v1)* for consistency. Percent identity was determined by first translating both the *AAD-2* and *tfdA* DNA sequences (SEQ ID NO:12 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively) to proteins (SEQ ID NO:13 of PCT/US2005/014737 and GENBANK Accession No. M16730, respectively), then using ClustalW in the VectorNTI software package to perform the multiple sequence alignment...

...*AAD-12 (v1)* and *AAD-2 (v1)* did provide detectable 2,4-D resistance versus the transformed and untransformed control lines; however, individual constructs were widely variable in their ability to impart 2,4-D resistance to individual T<sub>1</sub> *Arabidopsis* plants. Surprisingly, *AAD-2 (v1)* and *AAD-2 (v2)* transformants were far less resistant to 2,4-D than the *AAD-12 (v1)* gene, both from a frequency of highly tolerant plants as well as overall average injury. No plants transformed with *AAD-2 (v1)* survived 200 g ae/ha 2,4-D relatively uninjured (<20% visual injury), and overall population injury was about 83% (see PCT/US2005/014737). Conversely, *AAD-12 (v1)* had a population injury average of about 6% when treated with 3,200 g ae/ha 2,4-D (Table 11). Tolerance improved slightly for plant-optimized *AAD-2 (v2)* versus the native gene; however, comparison of both *AAD-12* and *AAD-2* plant optimized genes indicates a significant advantage for *AAD-12 (v1)* in planta.

These results are unexpected given that the *in vitro* comparison of *AAD-2 (v1)* (see PCT/US2005/014737) and *AAD-12 (v2)* indicated both were highly efficacious at degrading 2,4-D and both shared an S-type specificity with respect to chiral aryloxyalkanoate substrates. *AAD-2 (v1)* is expressed in individual T<sub>1</sub> plants to varying levels; however, little protection from 2,4-D injury is afforded by this expressed protein. No substantial difference was evident in protein expression level (*in planta*) for the native and plant optimized *AAD-2* genes (see PCT/US2005/014737). These data corroborate earlier findings that make the functional expression of *AAD-12 (v1)* *in planta*, and resulting herbicide resistance to 2,4-D and pyridyloxyacetate herbicides, unexpected.

Thus, it is clear that the subject *AAD-12* surprisingly performed much better in plants than *AAD-2* (which had little if any activity in plants), even though *AAD-2* had a higher level of identity to the cited *tfdA* gene/protein of Klaphammer.

The above results show that the behavior and activity of a herbicide-tolerance bacterial gene in plants cannot be predicted based on its behavior and activity in bacteria (the *in vitro* results were in *E. coli*). *AAD-2* is *more* related at the sequence level to the cited *tfdA* than is the subject *AAD-12*. While both genes/proteins were active in bacteria, *AAD-2* was essentially inactive in plants, and the subject *AAD-12* was surprisingly very active in plants.

This shows that herbicidal activity in plants is unpredictable and that the results in bacteria cannot readily be used to predict *in planta* behavior. Again, there were over a

USSN 12/091,896  
Attorney ref. DAS-P0129-US-02

hundred choices to begin with. If AAD-2 were used as a point of reference (again, AAD-2 is *more* related to tfdA at the sequence level than is AAD-12), one would have expected *no* activity in plants for AAD-12.

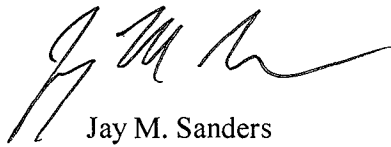
The foregoing should show that there was no reasonable prediction or expectation of successfully producing herbicide-tolerant plants using the subject AAD-12.

In light of these surprising and unexpected results, the withdrawal of this rejection for obviousness is respectfully requested.

The applicants believe that the subject application is in condition for allowance, and such action is respectfully requested. The undersigned invites the examiner to address any remaining issues in a telephone interview to expedite the prosecution of this application.

Any additional fees associated with this Response are hereby authorized to be charged to Baker & Daniels LLP's Deposit Account No. 02-0390.

Respectfully submitted,



Jay M. Sanders  
Registration No. 39,355  
Phone No.: 317 237 1245  
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Address: 300 N. Meridian Street  
Suite 2700  
Indianapolis, IN 46204

Date Submitted: October 14, 2011

Attachments: -replacement sequence listing (.txt file)  
-Statement regarding replacement sequence listing

# **Exhibit W**



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office  
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Alexandria, Virginia 22313-1450  
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
12/091,896	11/03/2008	Terry R. Wright	DAS-PO129-US-02	6428
83067	7590	03/27/2012	EXAMINER	
Faegre Baker Daniels LLP- Dow AgroSciences 300 North Meridian Street, Suite 2700 Indianapolis, IN 46204			KRUSE, DAVID H	
			ART UNIT	PAPER NUMBER
			1638	
			NOTIFICATION DATE	DELIVERY MODE
			03/27/2012	ELECTRONIC

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

inteas@faegrebd.com  
cynthia.payson@faegrebd.com

# 7774

**Office Action Summary**

Application No.

12/091,896

Applicant(s)

WRIGHT ET AL.

Examiner

DAVID H. KRUSE

Art Unit

1638

**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --****Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTH(S) OR THIRTY (30) DAYS, WHICHEVER IS LONGER, FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

- 1) ☒ Responsive to communication(s) filed on 14 October 2011 and 25 January 2012.
- 2a) ☒ This action is **FINAL**. 2b) ☐ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on \_\_\_\_; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims**

- 5) ☒ Claim(s) 89-103 is/are pending in the application.
- 5a) Of the above claim(s) \_\_\_\_ is/are withdrawn from consideration.
- 6) ☒ Claim(s) 89-102 is/are allowed.
- 7) ☒ Claim(s) 103 is/are rejected.
- 8) ☐ Claim(s) \_\_\_\_ is/are objected to.
- 9) ☐ Claim(s) \_\_\_\_ are subject to restriction and/or election requirement.

**Application Papers**

- 10) ☒ The specification is objected to by the Examiner.
- 11) ☐ The drawing(s) filed on \_\_\_\_ is/are: a) ☐ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).
- 12) ☐ The oath or declaration is objected to by the Examiner. Note the attached Office Action or form PTO-152.

**Priority under 35 U.S.C. § 119**

- 13) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).
- a) ☐ All b) ☐ Some \* c) ☐ None of:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

- |  |   |
|--|---|
| 1) <input type="checkbox"/> Notice of References Cited (PTO-892)                     | 4) <input type="checkbox"/> Interview Summary (PTO-413)           |
| 2) <input type="checkbox"/> Notice of Draftsperson's Patent Drawing Review (PTO-948) | Paper No(s)/Mail Date. ____.                                      |
| 3) <input type="checkbox"/> Information Disclosure Statement(s) (PTO/SB/08)          | 5) <input type="checkbox"/> Notice of Informal Patent Application |
| Paper No(s)/Mail Date ____.  | 6) <input type="checkbox"/> Other: ____.                          |



Application/Control Number: 12/091,896  
Art Unit: 1638

Page 2

### **DETAILED ACTION**

1. This Office action is in response to the Amendment filed 25 January 2012 and Remarks filed on 14 October 2011.
2. The specification is now in compliance with the Sequence Rules.
3. The rejection under 35 U.S.C. 101 is withdrawn in view of Applicants' amendment to claim 103.
4. The rejection under 35 U.S.C. 112, first paragraph, for lack of adequate written description is withdrawn in view of Applicants' amendments to the claims.
5. The rejection of claims 89-94 and 103 under 35 U.S.C. 102(b) as being anticipated by Kaphammer (U.S. Patent 5,608,147) taken with the evidence of Fourgoux-Nicol *et al* (1999, Plant Molecular Biology 40: 857-872) is withdrawn in view of Applicants' amendments to the claims.
6. The rejection of claim 89-102 under 35 U.S.C. 103(a) is withdrawn in view of Applicants' amendments to the claims and arguments as directed to the claims as presently amended.

### ***Specification***

7. The disclosure remains objected to because of the following informalities: On page 106 there is a Table that lacks a table number, subsequent Tables would need to be renumbered and the specification amended to reflect the renumbering of Tables. Applicants argue that the table on page 106 was already identified in the specification as filed as Table 26 at the top left corner of [the] table (page 5, 2<sup>nd</sup> paragraph of the

Application/Control Number: 12/091,896  
Art Unit: 1638

Page 3

Remarks). The specification of record has a Table 26 on page 105, the table on page 106 does not have a number.

Appropriate correction is required.

***Claim Rejections - 35 USC § 102***

8. The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office action:

A person shall be entitled to a patent unless –

(b) the invention was patented or described in a printed publication in this or a foreign country or in public use or on sale in this country, more than one year prior to the date of application for patent in the United States.

9. Claim 103 remains rejected under 35 U.S.C. § 102(b) as being anticipated by Schleinitz *et al* (September 2004, Applied and Environmental Microbiology 70(9): 5357-5365). This rejection is repeated for the reason of record as set forth in the last Office action mailed 14 July 2011. Applicant's arguments filed 14 October 2011 have been fully considered but they are not persuasive.

Schleinitz *et al* disclose an isolated polynucleotide comprising instant SEQ ID NO: 1 at GenBank accession number AY327575 (page 5359, right column, 5<sup>th</sup> paragraph). The encoded protein (see FIG 2 on page 5360) would inherently have aryloxyalkanoate dioxygenase activity. Hence, the claim limitation had been previously disclosed.

Applicants argue that Schleinitz relates to bacterial genes. Applicants argue that claim 103 specified that the claimed polynucleotide "comprises a codon composition that is optimized for expression in a plant." Applicants argue that Schleinitz does not teach the use of plant codons to increase expression of those genes (page 5, 6<sup>th</sup>

Application/Control Number: 12/091,896

Page 4

Art Unit: 1638

paragraph of the Remarks). This argument is not found to be persuasive because the claim fails to set forth any specific structure that would obviate a finding of anticipation. Since plant plastids will readily express bacterial genes, the limitation “optimized for expression in a plant” as presently stated has no patentable weight in the instant case. The prior art protein has a first amino acid methionine, and the protein sequence would anticipate all of the coding sequences that would produce it since one skilled in the art could readily envision them.

### ***Conclusion***

10. **THIS ACTION IS MADE FINAL.** Applicant is reminded of the extension of time policy as set forth in 37 CFR 1.136(a).

A shortened statutory period for reply to this final action is set to expire THREE MONTHS from the mailing date of this action. In the event a first reply is filed within TWO MONTHS of the mailing date of this final action and the advisory action is not mailed until after the end of the THREE-MONTH shortened statutory period, then the shortened statutory period will expire on the date the advisory action is mailed, and any extension fee pursuant to 37 CFR 1.136(a) will be calculated from the mailing date of the advisory action. In no event, however, will the statutory period for reply expire later than SIX MONTHS from the mailing date of this final action.

11. Claim 103 remains rejected.

12. Claims 89-102 are allowed.

13. Any inquiry concerning this communication or earlier communications from the examiner should be directed to David H. Kruse, Ph.D. whose telephone number is (571)

Application/Control Number: 12/091,896

Page 5

Art Unit: 1638

272-0799. The examiner can normally be reached on Monday to Friday from 8:00 a.m. to 4:30 p.m.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, Anne Marie Grunberg can be reached at (571) 272-0975. The central FAX number for official correspondence is 571-273-8300.

Any inquiry of a general nature or relating to the status of this application or proceeding should be directed to the Group Receptionist whose telephone number is (571) 272-1600.

/David H Kruse/  
Primary Examiner, Art Unit 1638

# **Exhibit X**



UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE  
United States Patent and Trademark Office  
Address: COMMISSIONER FOR PATENTS  
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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/223,249	03/24/2014	Nathan Bard	14764-246375	1147

107540 7590 09/08/2016  
Barnes & Thornburg LLP (Dow)  
11 South Meridian Street  
Indianapolis, IN 46204

EXAMINER
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KOVALENKO, MYKOLA V

ART UNIT	PAPER NUMBER
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1662

NOTIFICATION DATE	DELIVERY MODE
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09/08/2016

ELECTRONIC

**Please find below and/or attached an Office communication concerning this application or proceeding.**

The time period for reply, if any, is set in the attached communication.

Notice of the Office communication was sent electronically on above-indicated "Notification Date" to the following e-mail address(es):

INDocket@btlaw.com



Application No.  
14/223,249  
# 77181Applicant(s)  
BARD ET AL.**Office Action Summary**Examiner  
MYKOLA KOVALENKOArt Unit  
1662AIA (First Inventor to File)  
Status  
No**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address --****Period for Reply**

A SHORTENED STATUTORY PERIOD FOR REPLY IS SET TO EXPIRE 3 MONTHS FROM THE MAILING DATE OF THIS COMMUNICATION.

- Extensions of time may be available under the provisions of 37 CFR 1.136(a). In no event, however, may a reply be timely filed after SIX (6) MONTHS from the mailing date of this communication.
- If NO period for reply is specified above, the maximum statutory period will apply and will expire SIX (6) MONTHS from the mailing date of this communication.
- Failure to reply within the set or extended period for reply will, by statute, cause the application to become ABANDONED (35 U.S.C. § 133). Any reply received by the Office later than three months after the mailing date of this communication, even if timely filed, may reduce any earned patent term adjustment. See 37 CFR 1.704(b).

**Status**

- 1) ☒ Responsive to communication(s) filed on March 24, 2014.  
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on \_\_\_\_\_.
- 2a) ☐ This action is **FINAL**. 2b) ☒ This action is non-final.
- 3) ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on \_\_\_\_\_; the restriction requirement and election have been incorporated into this action.
- 4) ☐ Since this application is in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under *Ex parte Quayle*, 1935 C.D. 11, 453 O.G. 213.

**Disposition of Claims\***

- 5) ☒ Claim(s) 1-8 is/are pending in the application.  
5a) Of the above claim(s) 5-8 is/are withdrawn from consideration.
- 6) ☐ Claim(s) \_\_\_\_\_ is/are allowed.
- 7) ☒ Claim(s) 1-4 is/are rejected.
- 8) ☐ Claim(s) \_\_\_\_\_ is/are objected to.
- 9) ☐ Claim(s) \_\_\_\_\_ are subject to restriction and/or election requirement.

\* If any claims have been determined allowable, you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see [http://www.uspto.gov/patents/init\\_events/pph/index.jsp](http://www.uspto.gov/patents/init_events/pph/index.jsp) or send an inquiry to [PPHfeedback@uspto.gov](mailto:PPHfeedback@uspto.gov).

**Application Papers**

- 10) ☒ The specification is objected to by the Examiner.
- 11) ☒ The drawing(s) filed on March 24, 2014 is/are: a) ☒ accepted or b) ☐ objected to by the Examiner.  
Applicant may not request that any objection to the drawing(s) be held in abeyance. See 37 CFR 1.85(a).  
Replacement drawing sheet(s) including the correction is required if the drawing(s) is objected to. See 37 CFR 1.121(d).

**Priority under 35 U.S.C. § 119**

- 12) ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

- a) ☐ All b) ☐ Some\*\* c) ☐ None of the:
1. ☐ Certified copies of the priority documents have been received.
  2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
  3. ☐ Copies of the certified copies of the priority documents have been received in this National Stage application from the International Bureau (PCT Rule 17.2(a)).

\*\* See the attached detailed Office action for a list of the certified copies not received.

**Attachment(s)**

- 1) ☐ Notice of References Cited (PTO-892)
- 2) ☒ Information Disclosure Statement(s) (PTO/SB/08a and/or PTO/SB/08b)  
Paper No(s)/Mail Date \_\_\_\_\_
- 3) ☐ Interview Summary (PTO-413)  
Paper No(s)/Mail Date. \_\_\_\_\_
- 4) ☐ Other: \_\_\_\_\_

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 2

The present application is being examined under the pre-AIA first to invent provisions.

### **DETAILED ACTION**

1. Claims 1-8 are pending.
2. Claims 1-4 are examined herein.

### ***Election/Restrictions***

3. Restriction to one of the following inventions is required under 35 U.S.C. 121:

I. Claims 1-4, drawn to a polynucleotide comprising SEQ ID NO: 14; to a soybean plant and seed of soybean event 9582.814.19.1; and to an isolated polynucleotide diagnostic for the event, classified, for example, in CPC C12N 15/82.

II. Claim 5-8, drawn to a method of detecting the presence of soybean event 9582.814.19.1, classified, for example, in CPC C12Q 1/68.

4. The inventions are distinct, each from the other because of the following reasons:

Inventions I and II are related as product and process of use. The inventions can be shown to be distinct if either or both of the following can be shown: (1) the process for using the product as claimed can be practiced with another materially different product or (2) the product as claimed can be used in a materially different process of using that product. See MPEP § 806.05(h). In the instant case, the process of Invention II can be practiced with a product that is materially different from the product of Invention I. For example, the method of Invention II could be used to detect the absence of the soybean event of Invention I.

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 3

5. Restriction for examination purposes as indicated is proper because all these inventions listed in this action are independent or distinct for the reasons given above and there would be a serious search and/or examination burden if restriction were not required because at least the following reason(s) apply:

- (a) The inventions have acquired a separate status in the art in view of their different classification;
- (b) The inventions have acquired a separate status in the art due to their recognized divergent subject matter;
- (c) The inventions require a different field of search (for example, searching different classes/subclasses or electronic resources, or employing different search queries);
- (d) The prior art applicable to one invention would not likely be applicable to another invention;
- (e) The inventions are likely to raise different non-prior art issues under 35 U.S.C. 101 and/or 35 U.S.C. 112, first paragraph.

**Applicant is advised that the reply to this requirement to be complete must include (i) an election of an invention to be examined even though the requirement may be traversed (37 CFR 1.143) and (ii) identification of the claims encompassing the elected invention.**

The election of an invention may be made with or without traverse. To reserve a right to petition, the election must be made with traverse. If the reply does not distinctly and specifically point out supposed errors in the restriction requirement, the election

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 4

shall be treated as an election without traverse. Traversal must be presented at the time of election in order to be considered timely. Failure to timely traverse the requirement will result in the loss of right to petition under 37 CFR 1.144. If claims are added after the election, applicant must indicate which of these claims are readable upon the elected invention.

Should applicant traverse on the ground that the inventions are not patentably distinct, applicant should submit evidence or identify such evidence now of record showing the inventions to be obvious variants or clearly admit on the record that this is the case. In either instance, if the examiner finds one of the inventions unpatentable over the prior art, the evidence or admission may be used in a rejection under 35 U.S.C. 103(a) of the other invention.

6. The examiner has required restriction between product or apparatus claims and process claims. Where applicant elects claims directed to the product/apparatus, and all product/apparatus claims are subsequently found allowable, withdrawn process claims that include all the limitations of the allowable product/apparatus claims should be considered for rejoinder. All claims directed to a nonelected process invention must include all the limitations of an allowable product/apparatus claim for that process invention to be rejoined.

In the event of rejoinder, the requirement for restriction between the product/apparatus claims and the rejoined process claims will be withdrawn, and the rejoined process claims will be fully examined for patentability in accordance with 37 CFR 1.104. Thus, to be allowable, the rejoined claims must meet all criteria for

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 5

patentability including the requirements of 35 U.S.C. 101, 102, 103 and 112. Until all claims to the elected product/apparatus are found allowable, an otherwise proper restriction requirement between product/apparatus claims and process claims may be maintained. Withdrawn process claims that are not commensurate in scope with an allowable product/apparatus claim will not be rejoined. See MPEP § 821.04.

Additionally, in order for rejoinder to occur, applicant is advised that the process claims should be amended during prosecution to require the limitations of the product/apparatus claims. **Failure to do so may result in no rejoinder.** Further, note that the prohibition against double patenting rejections of 35 U.S.C. 121 does not apply where the restriction requirement is withdrawn by the examiner before the patent issues. See MPEP § 804.01.

7. Applicant is reminded that upon the cancellation of claims to a non-elected invention, the inventorship must be corrected in compliance with 37 CFR 1.48(a) if one or more of the currently named inventors is no longer an inventor of at least one claim remaining in the application. A request to correct inventorship under 37 CFR 1.48(a) must be accompanied by an application data sheet in accordance with 37 CFR 1.76 that identifies each inventor by his or her legal name and by the processing fee required under 37 CFR 1.17(i).

8. In the telephone communication on August 18, 2016, Applicant's representative Yonghao Hou informed the Examiner that Applicant elected, without traverse, Invention I, claims 1-4 for prosecution on the merits. Applicant's election is acknowledged. Claims 5-8 are withdrawn from further consideration pursuant to 37 CFR 1.142(b) as

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 6

being drawn to a nonelected invention, there being no allowable generic or linking claim.

Election was made **without** traverse in the telephonic communication on August 18, 2016.

### ***Claim Objections***

9. Claim 2 is objected to because of the following informalities: in claim 2, the term “event 814” should recite “event 9582.814.19.1.” Appropriate correction is required.

### ***Specification***

10. The disclosure is objected to because in lines 2-3 of paragraph 1, Applicant claims priority to US Provisional Applications No. 61/511,664 and 61/521,798.

Applicant cannot claim priority to a provisional application, only “benefit of” a provisional application.

The abstract is objected to because in line 1, the phrase “Soybean event 9582.814.19.1 comprises genes encoding” is grammatically incorrect. The phrase should either have the word “comprises” replaced with “comprising,” or the words “wherein the event comprises” inserted after “9582.814.19.1.”

Appropriate correction is required.

### ***Claim Rejections - 35 USC § 112 - Second Paragraph***

11. The following is a quotation of 35 U.S.C. 112(b):  
(b) CONCLUSION.—The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the inventor or a joint inventor regards as the invention.

The following is a quotation of 35 U.S.C. 112 (pre-AIA), second paragraph:  
The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.



Application/Control Number: 14/223,249  
Art Unit: 1662

Page 7

12. Claim 3 is rejected under 35 U.S.C. 112(b) or 35 U.S.C. 112 (pre-AIA), second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which the inventor or a joint inventor, or for pre-AIA the applicant regards as the invention.

At claim 3, due to the use of the conjunction “and” in the second to last line, before “10 base pairs of SEQ ID NO: 2” makes it unclear what is comprised in the recited isolated polynucleotide diagnostic for the event. The specification teaches that SEQ ID NO: 14 is the full-length sequence of the claimed event (paragraph 0039), yet the claim requires the presence of additional sequences.

In addition, the recitations “comprising base pairs ... in each direction from the ... junction” are unclear. It is unclear, for example, whether the phrase “at least 10 base pairs in length comprising 1400/1401 of SEQ ID NO: 1 in each direction from the base pairs 1400/1401 junction” requires that the DNA molecule encompass at least 10 or at least 20 base pairs. It is also unclear whether the polynucleotide “comprising 1400/1401 of SEQ ID NO: 1” comprises both, nucleotides 1400 and 1401, or only one of them. The metes and bounds of the claim are unclear.

***Claim Rejections - 35 USC § 112 - Fourth Paragraph***

13. The following is a quotation of 35 U.S.C. 112(d):

(d) REFERENCE IN DEPENDENT FORMS.—Subject to subsection (e), a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

The following is a quotation of pre-AIA 35 U.S.C. 112, fourth paragraph:

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 8

Subject to the following paragraph [i.e., the fifth paragraph of pre-AIA 35 U.S.C. 112], a claim in dependent form shall contain a reference to a claim previously set forth and then specify a further limitation of the subject matter claimed. A claim in dependent form shall be construed to incorporate by reference all the limitations of the claim to which it refers.

14. Claim 4 is rejected under 35 U.S.C. 112(d) or pre-AIA 35 U.S.C. 112, 4th paragraph, as being of improper dependent form for failing to further limit the subject matter of the claim upon which it depends, or for failing to include all the limitations of the claim upon which it depends.

Claim 4 fails to include all the limitations of claim 3 from which it depends. Claims 3 was found indefinite, as set forth above. For the purpose of this rejection, in view of the teachings of the specification, claim 3 is read as reciting the nucleic acid sequences in the alternative (see Brief Description of Sequences on pg. 7-8). Under this interpretation, claim 4 could be infringed by a polynucleotide that would not infringe on claim 3. For example, a nucleic acid comprising base pairs 1200-1600 of SEQ ID NO: 2 would not read on a number of nucleic acids recited in claim 1. See MPEP 608.01(n).

Applicant may cancel the claim(s), amend the claim(s) to place the claim(s) in proper dependent form, rewrite the claim(s) in independent form, or present a sufficient showing that the dependent claim(s) complies with the statutory requirements.

***Claim Rejections - 35 USC § 112 - First Paragraph***

15. The following is a quotation of the first paragraph of 35 U.S.C. 112(a):

(a) IN GENERAL.—The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor or joint inventor of carrying out the invention.

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 9

The following is a quotation of the first paragraph of pre-AIA 35 U.S.C. 112:

The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

16. Claim 2 is rejected under 35 U.S.C. 112(a) or 35 U.S.C. 112 (pre-AIA), first paragraph, as failing to comply with the enablement requirement. The claim(s) contains subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention.

The claim is directed to novel plants. Since the plants are essential to the claimed invention, they must be obtainable by a repeatable method set forth in the specification or otherwise be readily available to the public. If the plants are not so obtainable or available, a deposit of the plant may satisfy the requirements of 35 USC 112. A deposit of 2,500 seeds of each of the claimed embodiments is considered sufficient to ensure public availability. The specification does not disclose a repeatable process to obtain the plants and it is not apparent whether the plants are readily available to the public. It is noted that Applicants have deposited the plants with the American Type Tissue Culture Collection under Accession Number PTA-12006, but there is no affirmative statement as to irrevocable public availability (see pg. 6, paragraph 0022 for the Deposit Statement).

If a deposit is made under the terms of the Budapest Treaty, then a statement, affidavit or declaration by Applicants, or a statement by an attorney of record over his or

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 10

her signature and registration number, or someone empowered to make such a statement, stating that the instant invention will be irrevocably and without restriction released to the public upon the issuance of a patent, would satisfy the deposit requirement made herein.

If a deposit has not been made under the Budapest Treaty, then in order to certify that the deposit meets the criteria set forth in 37 CFR 1.801-1.809 and MPEP 2402-2411.05, Applicant may provide assurance of compliance by statement, affidavit or declaration, or by someone empowered to make the same, or by a statement by an attorney of record over his or her signature and registration number showing that:

- (a) during the pendency of this application, access to the invention will be afforded to the Commissioner upon request;
- (b) all restrictions upon availability to the public will be irrevocably removed upon granting of the patent;
- (c) the deposit will be maintained in a public depository for a period of 30 years or 5 years after the last request or for the enforceable life of the patent, whichever is longer;
- (d) a test of the viability of the biological material at the time of deposit (see 37 CFR 1.807); and,
- (e) the deposit will be replaced if it should ever become inviable.

In addition, the identifying information set forth in 37 CFR 1.809(d) should be added to the specification. See 37 CFR 1.801 - 1.809 [MPEP 2401-2411.05] for additional explanation of these requirements.

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 11

### ***Double Patenting***

17. A rejection based on double patenting of the “same invention” type finds its support in the language of 35 U.S.C. 101 which states that “whoever invents or discovers any new and useful process... may obtain a patent therefor...” (Emphasis added). Thus, the term “same invention,” in this context, means an invention drawn to identical subject matter. See *Miller v. Eagle Mfg. Co.*, 151 U.S. 186 (1894); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Ockert*, 245 F.2d 467, 114 USPQ 330 (CCPA 1957).

A statutory type (35 U.S.C. 101) double patenting rejection can be overcome by canceling or amending the claims that are directed to the same invention so they are no longer coextensive in scope. The filing of a terminal disclaimer cannot overcome a double patenting rejection based upon 35 U.S.C. 101.

18. Claim 2 is rejected under 35 U.S.C. 101 as claiming the same invention as that of claim 7 of prior U.S. Patent No. 8,680,363. The instant claim is directed to a soybean plant, seed, or other plant part of said plant comprising “event 814” in seed deposited with the ATCC under Accession No. PTA-12006. Claim 7 of the patent is drawn to a soybean plant, wherein the representative seed of said plant has been deposited with the ATCC under Accession NO. PTA-12006. The plant and seed of claim 7 of the patent would inherently comprise a plant part recited in the instant claim. As a result, the two claims are drawn to the same invention. This is a statutory double patenting rejection.

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 12

19. The nonstatutory double patenting rejection is based on a judicially created doctrine grounded in public policy (a policy reflected in the statute) so as to prevent the unjustified or improper timewise extension of the “right to exclude” granted by a patent and to prevent possible harassment by multiple assignees. A nonstatutory double patenting rejection is appropriate where the conflicting claims are not identical, but at least one examined application claim is not patentably distinct from the reference claim(s) because the examined application claim is either anticipated by, or would have been obvious over, the reference claim(s). See, e.g., *In re Berg*, 140 F.3d 1428, 46 USPQ2d 1226 (Fed. Cir. 1998); *In re Goodman*, 11 F.3d 1046, 29 USPQ2d 2010 (Fed. Cir. 1993); *In re Longi*, 759 F.2d 887, 225 USPQ 645 (Fed. Cir. 1985); *In re Van Ornum*, 686 F.2d 937, 214 USPQ 761 (CCPA 1982); *In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *In re Thorington*, 418 F.2d 528, 163 USPQ 644 (CCPA 1969).

A timely filed terminal disclaimer in compliance with 37 CFR 1.321(c) or 1.321(d) may be used to overcome an actual or provisional rejection based on nonstatutory double patenting provided the reference application or patent either is shown to be commonly owned with the examined application, or claims an invention made as a result of activities undertaken within the scope of a joint research agreement. See MPEP § 717.02 for applications subject to examination under the first inventor to file provisions of the AIA as explained in MPEP § 2159. See MPEP §§ 706.02(l)(1) - 706.02(l)(3) for applications not subject to examination under the first inventor to file



Application/Control Number: 14/223,249  
Art Unit: 1662

Page 13

provisions of the AIA. A terminal disclaimer must be signed in compliance with 37 CFR 1.321(b).

The USPTO Internet website contains terminal disclaimer forms which may be used. Please visit [www.uspto.gov/patent/patents-forms](http://www.uspto.gov/patent/patents-forms). The filing date of the application in which the form is filed determines what form (e.g., PTO/SB/25, PTO/SB/26, PTO/AIA/25, or PTO/AIA/26) should be used. A web-based eTerminal Disclaimer may be filled out completely online using web-screens. An eTerminal Disclaimer that meets all requirements is auto-processed and approved immediately upon submission. For more information about eTerminal Disclaimers, refer to [www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp](http://www.uspto.gov/patents/process/file/efs/guidance/eTD-info-I.jsp).

20. Claims 1, 3, and 4 are rejected on the ground of nonstatutory double patenting as being unpatentable over claims 1-9 of U.S. Patent No. 8,680,363. Although the claims at issue are not identical, they are not patentably distinct from each other because the claims of the patent make obvious the invention of the instant claims.

The instant claims are drawn to a polynucleotide comprising SEQ ID NO: 14 and isolated polynucleotides that include portions of SEQ ID NO: 1 or 2. The claims of the patent are drawn to a soybean plant and seed comprising SEQ ID NO: 14, and the methods of using said plant that requires SEQ ID NO: 14. The instant specification teaches that SEQ ID NO: 14 is the full length sequence of soybean event 9582.814.9.1 and thus comprises SEQ ID NO: 1 and 2, which are 3' and 5' flanking sequences (see Brief Description of The Sequences on pg. 7-8). SEQ ID NO: 14 of the instant claims

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 14

appears identical to SEQ ID NO: 14 of the patent and thus the claims of the patent would make obvious the instant invention.

21. Claims 3 and 4 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claims 6, 8, 9, and 13 of copending Application No. 14/234,923 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because of the overlap in the claimed subject matter. The instant claims are directed to isolated polynucleotides that are diagnostic for soybean event 95871.814.19.1, comprise fragments of SEQ ID NO: 1 or 2, and encompass the junction, or comprise full-length SEQ ID NO: 1 or 2. The claims of the co-pending application are directed to an isolated DNA molecule that would read on the instantly recited portions of SEQ ID NO: 1 or 2. SEQ ID NO: 1 and 2 of the instant claims have 100% identity to SEQ ID NO: 1 and 2 of the co-pending application. Thus the claims of the co-pending application make obvious the invention of instant claims 3 and 4.

This is a provisional nonstatutory double patenting rejection because the patentably indistinct claims have not in fact been patented.

22. Claims 3 and 4 are provisionally rejected on the ground of nonstatutory double patenting as being unpatentable over claim 12 of copending Application No. 14/784,044 (reference application). Although the claims at issue are not identical, they are not patentably distinct from each other because of the overlap in the claimed subject matter. The instant claims are directed to an isolated polynucleotide that is diagnostic for

Application/Control Number: 14/223,249

Page 15

Art Unit: 1662

soybean event 95871.814.19.1 that comprise fragments of SEQ ID NO: 1 or 2 and encompass the junction, or comprise full-length SEQ ID NO: 1 or 2. The claim of the co-pending application are directed to a composition comprising a Bt toxin encoded by a polynucleotide, which polynucleotide reads on the instantly recited portions of SEQ ID NO: 1 or 2. SEQ ID NO: 1 and 2 of the instant claims have 100% identity to SEQ ID NO: 1 and 2 of the co-pending application. Thus the claims of the co-pending application make obvious the invention of instant claims 3 and 4.

### ***Contact Information***

23. No claims are allowed.

24. The claims appear free of the art, due to the failure of the prior art to either teach or reasonably suggest the plants comprising event 9582.814.19.1, the polynucleotide of the instant SEQ ID NO: 14, SEQ ID NO: 1 or 2, or the fragments of SEQ ID NO: 1 or 2 that are diagnostic for the event and encompass the junction.

25. Any inquiry concerning this communication or earlier communications from the examiner should be directed to MYKOLA KOVALENKO whose telephone number is (571)272-6921. The examiner can normally be reached on Monday-Friday 9:30 am - 6 pm PST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor ZHOU (JOE) SHUBO can be reached at (571) 272-0724. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 16

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/MYKOLA KOVALENKO/  
Primary Examiner, Art Unit 1662

# **Exhibit Y**

**BARNES & THORNBURG LLP**

11 South Meridian Street  
Indianapolis, Indiana 46204  
(317) 236-1313  
(317) 231-7433 Fax

***IN THE UNITED STATES PATENT AND TRADEMARK OFFICE***

<i>Group:</i>	1662	}
		}
<i>Confirmation No.:</i>	1147	}
		}
<i>Application No.:</i>	14/223,249	}
		}
<i>Invention:</i>	INSECT RESISTANT AND	}
	HERBICIDE TOLERANT	}
	SOYBEAN EVENT	}
	9582.814.19.1	}
		}
<i>Inventor:</i>	Nathan Bard, et al.	}
		}
<i>Filed:</i>	March 24, 2014	}
		}
<i>Attorney</i>		}
<i>Docket:</i>	14764-246375	}
		}
<i>Examiner:</i>	KOVALENKO, MYKOLA V.	}

ELECTRONICALLY FILED:  
December 8, 2016

**RESPONSE AND AMENDMENT UNDER 37 C.F.R. § 1.111**

Mail Stop Amendment  
Commissioner for Patents  
P. O. Box 1450  
Alexandria, Virginia 22313-1450

Sir:

In response to the Office Action mailed September 8, 2016, Applicant requests entry of the amendments and consideration of the accompanying remarks. Applicant believes that no fees are required with this response. However, if any fees are required, the Director is hereby authorized



U.S. Application Serial No. 14/223,249

to charge those fees to the account of Barnes & Thornburg LLP, Deposit Account No. 10-0435, with reference to our matter 14764-246375.

**Amendments to the Abstract** begin on page 3 of this paper.

**Amendments to the Specification** begin on page 4 of this paper.

**Amendments to the Claims** begin on page 5 of this paper.

**Remarks** begin on page 7 of this paper.

U.S. Application Serial No. 14/223,249

**AMENDMENTS TO THE ABSTRACT**

Please amend that abstract as follows:

Soybean event 9582.814.19.1 ~~comprises~~ comprising genes encoding Cry1F, Cry1Ac (synpro), and PAT, affording insect resistance and herbicide tolerance to soybean crops containing the event, and enabling methods for crop protection and protection of stored products.

U.S. Application Serial No. 14/223,249

**AMENDMENTS TO THE SPECIFICATION**

On page 1, please amend paragraph [0001] after the section heading of “Cross-Reference to Related Applications,” as follows:

**Cross-Reference to Related Applications**

This application is a continuation of U.S. application Ser. No. 13/559,177, filed Jul. 26, 2012, which claims ~~priority to~~ benefit of Provisional Application No. 61/511,664, filed Jul. 26, 2011, and Provisional Application No. 61/521,798, filed Aug. 10, 2011, all of which are herein incorporated by reference in their entireties.

U.S. Application Serial No. 14/223,249

### AMENDMENTS TO THE CLAIMS

A complete listing of the pending claims is provided pursuant to 37 C.F.R. §

1.121(c)(1). Please amend claims 2, 3, and 4 as follows:

1. (Original) A polynucleotide comprising SEQ ID NO:14.

2. (Currently amended) A soybean plant, seed, or other part of said plant comprising the polynucleotide of claim 1 ~~event 814 as present in seed deposited with the American Type Culture Collection under Accession No. PTA 12006.~~

3. (Currently amended) An isolated polynucleotide that is diagnostic for soybean event 9582.814.19.1, wherein said polynucleotide ~~comprises~~ is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:14, ~~DNA molecules at least 25 base pairs in length comprising base pairs 1400/1401 of SEQ ID NO:1 in each direction from the base pairs 1400/1401 junction; DNA molecules at least 10 base pairs in length comprising 1400/1401 of SEQ ID NO:1 in each direction from the base pairs 1400/1401 junction; amplicons at least 25 base pairs in length comprising 152-153 of SEQ ID NO:2 and at least 10 base pairs of SEQ ID NO:2 in each direction from the base pairs 152/153 junction.~~

4. (Currently amended) The isolated polynucleotide in claim 3 comprising one or more sequences selected from ~~from~~ the group consisting of base pairs 1385-1415 of SEQ ID NO:1, base pairs 1350-1450 of SEQ ID NO:1, base pairs 1300-1500 of SEQ ID NO:1, base pairs 1200-1600 of SEQ ID NO:1, base pairs 137-168 of SEQ ID NO:2, base pairs 103-203 of SEQ ID NO:2, and base pairs 3-303 of SEQ ID NO:2, ~~and SEQ ID NO:14.~~

5. (Withdrawn) A method of detecting the presence of soybean event 9582.814.19.1 in a sample, said method comprising: a. providing a polynucleotide probe selected from the group consisting of base pairs 1385-1415 of SEQ ID NO:1, base pairs 1350-1450 of SEQ ID NO:1, base pairs 1300-1500 of SEQ ID NO:1, base pairs 1200-1600 of SEQ ID NO:1, base pairs 137-168 of

U.S. Application Serial No. 14/223,249

SEQ ID NO:2, base pairs 103-203 of SEQ ID NO:2, base pairs 3-303 of SEQ ID NO:2, and SEQ ID NO:14 or their complements thereof; b. isolating the genomic DNA of the sample; c. conducting stringent hybridization assay for the polynucleotide probe in step a and the DNA in step b; and d. identifying positive hit as the presence of soybean event 9582.814.19.1 in the sample.

6. (Withdrawn) A method of detecting the presence of soybean event 9582.814.19.1 in a sample, comprising conducting a PCR amplification of a. partial or all of the flanking sequence, and b. partial or all of the insert sequence of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:14.

7. (Withdrawn) The method of claim 6, wherein the first primer is a polynucleotide at least 10 base pairs in length that selectively binds to a flanking sequence within residues 1-1400 of SEQ ID NO:1 or the complement thereof, and the second primer is selected from a polynucleotide at least 10 base pairs in length that selectively binds to an insert sequence within residues 1401-1836 of SEQ ID NO:1.

8. (Withdrawn) The method of claim 6, wherein the first primer is a polynucleotide at least 10 base pairs in length that selectively binds to an insert sequence within residues 1-152 of SEQ ID NO:2 or the complement thereof, and the second primer is selected from a polynucleotide at least 10 base pairs in length that selectively binds to a flanking sequence within residues 153-1550 of SEQ ID NO:2.

U.S. Application Serial No. 14/223,249

## **REMARKS**

### **I. Claim Amendments**

Claims 2, 3, and 4 have been amended. Support for the amendments is found throughout the application as filed and in the originally filed claims. Applicant respectfully requests entry of the amendments.

### **II. Election/Restriction**

Applicant affirms the election of the Group I claims (claims 1-4), as the Examiner discussed over the phone with Attorney Yonghao Hou on August 18, 2016.

### **III. Objection to the Claims**

The Examiner has objected to claim 2, requesting that the term “event 814” be replaced with the term “event 9582.814.19.1”. Claim 2 has been amended to remove the event language. Thus, the Examiner’s objection has been rendered moot. Withdrawal of the objection to claim 2 is respectfully requested.

### **IV. Objection to the Specification**

(1) The Examiner has objected to the specification at page 1, paragraph [0001]. The Examiner has requested that the phrase “priority to” be replaced with “benefit of”. Applicant has amended the specification accordingly. Withdrawal of the objection to the specification is respectfully requested.

(2) The Examiner has objected to the abstract. The Examiner has requested that the term “comprises” be replaced with “comprising.” Applicant has amended the abstract accordingly. Withdrawal of the objection to the specification is respectfully requested.



U.S. Application Serial No. 14/223,249

**V. Rejection of Claim 3 under 35 U.S.C. § 112, ¶ 2**

The Examiner has rejected claim 3 under 35 U.S.C. § 112, 2<sup>nd</sup> paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter applicant regards as the invention. The Examiner contends that it is unclear what is comprised in the recited isolated polynucleotide diagnostic for the event. The Examiner further contends that it is unclear whether the polynucleotide “comprising 1400/1401 of SEQ ID NO: 1” comprises both, nucleotides 1400 and 1401, or only one of them.”

Applicant has amended claim 3 to delete the language “DNA molecules at least 25 base pairs in length comprising base pairs 1400/1401 of SEQ ID NO:1 in each direction from the base pairs 1400/1401 junction; DNA molecules at least 10 base pairs in length comprising 1400/1401 of SEQ ID NO:1 in each direction from the base pairs 1400/1401 junction; amplicons at least 25 base pairs in length comprising 152-153 of SEQ ID NO:2 and at least 10 base pairs of SEQ ID NO:2 in each direction from the base pairs 152/153 junction.” Thus, claim 3 as amended recites “[a]n isolated polynucleotide that is diagnostic for soybean event 9582.814.19.1, wherein said polynucleotide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:14.” Therefore, the recited isolated polynucleotide diagnostic for the event is clear. In addition, the language pointed to by the Examiner has been removed from the claim. Accordingly, withdrawal of the rejection of claim 3 under 35 U.S.C. § 112, 2<sup>nd</sup> paragraph, is respectfully requested.

**VI. Rejection of Claim 4 under 35 U.S.C. § 112, ¶ 4**

The Examiner has rejected claim 4 under 35 U.S.C. § 112, 4<sup>th</sup> paragraph, arguing that the claim does not further limit the subject matter claimed. As discussed above, claim 3 has been amended to delete the language “DNA molecules at least 25 base pairs in length comprising

U.S. Application Serial No. 14/223,249

base pairs 1400/1401 of SEQ ID NO:1 in each direction from the base pairs 1400/1401 junction; DNA molecules at least 10 base pairs in length comprising 1400/1401 of SEQ ID NO:1 in each direction from the base pairs 1400/1401 junction; amplicons at least 25 base pairs in length comprising 152-153 of SEQ ID NO:2 and at least 10 base pairs of SEQ ID NO:2 in each direction from the base pairs 152/153 junction.”. Therefore, claim 3 as amended recites “[a]n isolated polynucleotide that is diagnostic for soybean event 9582.814.19.1, wherein said polynucleotide is selected from the group consisting of SEQ ID NO:1, SEQ ID NO:2, and SEQ ID NO:14.” In addition, claim 4 has been amended to delete the phrase “SEQ ID NO:14”. Thus, claim 4 further limits the subject matter of claim 3 from which it depends. Withdrawal of the rejection of claim 4 is respectfully requested.

#### **VII. Rejection of Claim 2 under 35 U.S.C. § 112, ¶ 1 - Enablement**

The Examiner has rejected claim 2 under 35 USC 112, 1<sup>st</sup> paragraph, as failing to comply with the enablement requirement. The Examiner contends that the specification does not disclose a repeatable process to obtain the plants and it is not apparent whether the plants are readily available to the public. Applicant has amended claim 2 to delete the phrase “event 814 as present in seed deposited with the American Type Culture Collection under Accession No. PTA-12006”. Thus, claim 2 as amended recites “[a] soybean plant, seed, or other part of said plant comprising the polynucleotide of claim 1.” Accordingly, reconsideration and withdrawal of the rejections under 35 U.S.C. § 112, first paragraph, are requested.

#### **VIII. Statutory Double Patenting under 35 U.S.C. § 101**

The Examiner has rejected claim 2 under 35 U.S.C. § 101 as claiming the same invention as that of claim 7 of prior U.S. Patent No. 8,680,363. Claim 7 of U.S. Patent No.

U.S. Application Serial No. 14/223,249

8,680,363 recites “[a] soybean plant, wherein representative seed of said soybean plant has been deposited with the American Type Culture Collection under Accession No. PTA-12006”.

Applicant has amended claim 2 to delete the phrase “event 814 as present in seed deposited with the American Type Culture Collection under Accession No. PTA-12006”. Thus, claim 2 as amended recites “[a] soybean plant, seed, or other part of said plant comprising the polynucleotide of claim 1.” Accordingly, reconsideration and withdrawal of the rejection under 35 U.S.C. § 101 is respectfully requested.

#### **IX. Non-Statutory Obviousness-Type Double Patenting**

(1) The Examiner has rejected claims 1, 3, and 4 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 1-9 of issued U.S. Patent No. 8,680,363. Transmitted herewith is a terminal disclaimer document under 37 C.F.R. § 1.321 disclaiming any term beyond the expiration date of U.S. Patent No. 8,680,363, and stating the assignees’ interest in the application. Thus, withdrawal of the obviousness-type double patenting rejection of claims 1, 3, and 4 over claims 1-9 of U.S. Patent No. 8,680,363 is respectfully requested.

(2) The Examiner has provisionally rejected claims 3 and 4 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claims 6, 8, 9, and 13 of copending U.S. Patent Application No. 14/234,923. Transmitted herewith is a terminal disclaimer document under 37 C.F.R. § 1.321 disclaiming any term beyond the expiration date of any patent that issues from U.S. Patent Application No. 14/234,923, and stating the assignees’ interest in the application. Thus, withdrawal of the obviousness-type double patenting rejection of claims 3 and 4

U.S. Application Serial No. 14/223,249

over claims 6, 8, 9, and 13 of copending U.S. Patent Application No. 14/234,923 is respectfully requested.

(3) The Examiner has provisionally rejected claims 3 and 4 on the ground of nonstatutory obviousness-type double patenting as being unpatentable over claim 12 of copending U.S. Patent Application No. 14/784,044. Applicant notes that “before consideration can be given to the issue of double patenting, two or more patents or applications must have at least one common inventor, common applicant, and/or be commonly assigned/owned or non-commonly assigned/owned but subject to a joint research agreement as set forth in 35 U.S.C. 102(c) or in pre-AIA 35 U.S.C. 103(c)(2) and (3).” See MPEP § 804.

The listed inventors for the ‘044 application are Elke Hellwege and Koen Van Den Eynde. The ‘044 application has been assigned to BAYER CROPSCIENCE AKTIENGESELLSCHAFT. BAYER CROPSCIENCE AKTIENGESELLSCHAFT is also the listed Applicant for the ‘044 application.

The listed inventors for the present application are Nathan Bard, Gregory A. Bradfish, Yunxing C. Cui, James E. Dripps, Thomas Hoffman, Dayakar Pareddy, Dawn M. Parkhurst, Sandra G. Toledo, Barry Wiggins, and Ning Zhou. The present application has been assigned to DOW AGROSCIENCES LLC. DOW AGROSCIENCES LLC is also the listed Applicant for the present application.

Thus, the present application and U.S. Patent Application No. 14/784,044 do **not** share at least one common inventor or common applicant, and/or are not commonly assigned/owned or non-commonly assigned/owned but subject to a joint research agreement as set forth in 35 U.S.C. 102(c) or in pre-AIA 35 U.S.C. 103(c)(2) and (3). Accordingly, the present rejection for

U.S. Application Serial No. 14/223,249

nonstatutory obviousness-type double patenting is improper. Withdrawal of the rejection is respectfully requested.

**CONCLUSION**

The foregoing amendments and remarks are believed to fully respond to the Examiner's rejections.

Respectfully submitted,  
BARNES & THORNBURG LLP

/Elizabeth Lehr Stetzer/  
Elizabeth Lehr Stetzer  
Agent Reg. No. 63,496

Indianapolis, Indiana 46204  
(317) 231-7228

# **Exhibit Z**





## UNITED STATES PATENT AND TRADEMARK OFFICE

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P.O. Box 1450  
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www.uspto.gov

## NOTICE OF ALLOWANCE AND FEE(S) DUE

107540 7590 03/07/2017  
Barnes & Thornburg LLP (Dow)  
11 South Meridian Street  
Indianapolis, IN 46204

EXAMINER

KOVALENKO, MYKOLA V

ART UNIT

PAPER NUMBER

1662

DATE MAILED: 03/07/2017

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
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14/223,249

03/24/2014

Nathan Bard

14764-246375

1147

TITLE OF INVENTION: INSECT RESISTANT AND HERBICIDE TOLERANT SOYBEAN EVENT 9582.814.19.1

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$960	\$0	\$0	\$960	06/07/2017

**THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED. THIS NOTICE OF ALLOWANCE IS NOT A GRANT OF PATENT RIGHTS. THIS APPLICATION IS SUBJECT TO WITHDRAWAL FROM ISSUE AT THE INITIATIVE OF THE OFFICE OR UPON PETITION BY THE APPLICANT. SEE 37 CFR 1.313 AND MPEP 1308.**

**THE ISSUE FEE AND PUBLICATION FEE (IF REQUIRED) MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED. SEE 35 U.S.C. 151. THE ISSUE FEE DUE INDICATED ABOVE DOES NOT REFLECT A CREDIT FOR ANY PREVIOUSLY PAID ISSUE FEE IN THIS APPLICATION. IF AN ISSUE FEE HAS PREVIOUSLY BEEN PAID IN THIS APPLICATION (AS SHOWN ABOVE), THE RETURN OF PART B OF THIS FORM WILL BE CONSIDERED A REQUEST TO REAPPLY THE PREVIOUSLY PAID ISSUE FEE TOWARD THE ISSUE FEE NOW DUE.**

**HOW TO REPLY TO THIS NOTICE:**

I. Review the ENTITY STATUS shown above. If the ENTITY STATUS is shown as SMALL or MICRO, verify whether entitlement to that entity status still applies.

If the ENTITY STATUS is the same as shown above, pay the TOTAL FEE(S) DUE shown above.

If the ENTITY STATUS is changed from that shown above, on PART B - FEE(S) TRANSMITTAL, complete section number 5 titled "Change in Entity Status (from status indicated above)".

For purposes of this notice, small entity fees are 1/2 the amount of undiscounted fees, and micro entity fees are 1/2 the amount of small entity fees.

II. PART B - FEE(S) TRANSMITTAL, or its equivalent, must be completed and returned to the United States Patent and Trademark Office (USPTO) with your ISSUE FEE and PUBLICATION FEE (if required). If you are charging the fee(s) to your deposit account, section "4b" of Part B - Fee(s) Transmittal should be completed and an extra copy of the form should be submitted. If an equivalent of Part B is filed, a request to reapply a previously paid issue fee must be clearly made, and delays in processing may occur due to the difficulty in recognizing the paper as an equivalent of Part B.

III. All communications regarding this application must give the application number. Please direct all communications prior to issuance to Mail Stop ISSUE FEE unless advised to the contrary.

**IMPORTANT REMINDER: Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.**

PART B - FEE(S) TRANSMITTAL

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Complete and send this form, together with applicable fee(s), to: **Mail** **Mail Stop ISSUE FEE**  
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107540 7590 03/07/2017  
**Barnes & Thornburg LLP (Dow)**  
**11 South Meridian Street**  
**Indianapolis, IN 46204**

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I hereby certify that this Fee(s) Transmittal is being deposited with the United States Postal Service with sufficient postage for first class mail in an envelope addressed to the Mail Stop ISSUE FEE address above, or being facsimile transmitted to the USPTO (571) 273-2885, on the date indicated below.

(Depositor's name)
(Signature)
(Date)

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/223,249	03/24/2014	Nathan Bard	14764-246375	1147

TITLE OF INVENTION: INSECT RESISTANT AND HERBICIDE TOLERANT SOYBEAN EVENT 9582.814.19.1

APPLN. TYPE	ENTITY STATUS	ISSUE FEE DUE	PUBLICATION FEE DUE	PREV. PAID ISSUE FEE	TOTAL FEE(S) DUE	DATE DUE
nonprovisional	UNDISCOUNTED	\$960	\$0	\$0	\$960	06/07/2017

EXAMINER	ART UNIT	CLASS-SUBCLASS
KOVALENKO, MYKOLA V	1662	800-265000

1. Change of correspondence address or indication of "Fee Address" (37 CFR 1.363).

☐ Change of correspondence address (or Change of Correspondence Address form PTO/SB/122) attached.

☐ "Fee Address" indication (or "Fee Address" Indication form PTO/SB/47; Rev 03-02 or more recent) attached. **Use of a Customer Number is required.**

2. For printing on the patent front page, list

(1) The names of up to 3 registered patent attorneys or agents OR, alternatively,

1 \_\_\_\_\_

(2) The name of a single firm (having as a member a registered attorney or agent) and the names of up to 2 registered patent attorneys or agents. If no name is listed, no name will be printed.

2 \_\_\_\_\_

3 \_\_\_\_\_

3. ASSIGNEE NAME AND RESIDENCE DATA TO BE PRINTED ON THE PATENT (print or type)

PLEASE NOTE: Unless an assignee is identified below, no assignee data will appear on the patent. If an assignee is identified below, the document has been filed for recordation as set forth in 37 CFR 3.11. Completion of this form is NOT a substitute for filing an assignment.

(A) NAME OF ASSIGNEE

(B) RESIDENCE: (CITY and STATE OR COUNTRY)

Please check the appropriate assignee category or categories (will not be printed on the patent): ☐ Individual ☐ Corporation or other private group entity ☐ Government

4a. The following fee(s) are submitted:

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☐ Publication Fee (No small entity discount permitted)

☐ Advance Order - # of Copies \_\_\_\_\_

4b. Payment of Fee(s): (Please first reapply any previously paid issue fee shown above)

☐ A check is enclosed.

☐ Payment by credit card. Form PTO-2038 is attached.

☐ The director is hereby authorized to charge the required fee(s), any deficiency, or credits any overpayment, to Deposit Account Number \_\_\_\_\_ (enclose an extra copy of this form).

5. **Change in Entity Status** (from status indicated above)

☐ Applicant certifying micro entity status. See 37 CFR 1.29

☐ Applicant asserting small entity status. See 37 CFR 1.27

☐ Applicant changing to regular undiscounted fee status.

**NOTE:** Absent a valid certification of Micro Entity Status (see forms PTO/SB/15A and 15B), issue fee payment in the micro entity amount will not be accepted at the risk of application abandonment.

**NOTE:** If the application was previously under micro entity status, checking this box will be taken to be a notification of loss of entitlement to micro entity status.

**NOTE:** Checking this box will be taken to be a notification of loss of entitlement to small or micro entity status, as applicable.

NOTE: This form must be signed in accordance with 37 CFR 1.31 and 1.33. See 37 CFR 1.4 for signature requirements and certifications.

Authorized Signature \_\_\_\_\_

Date \_\_\_\_\_

Typed or printed name \_\_\_\_\_

Registration No. \_\_\_\_\_



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APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
14/223,249	03/24/2014	Nathan Bard	14764-246375	1147

107540 7590 03/07/2017  
 Barnes & Thornburg LLP (Dow)  
 11 South Meridian Street  
 Indianapolis, IN 46204

EXAMINER

KOVALENKO, MYKOLA V

ART UNIT	PAPER NUMBER
----------	--------------

1662

DATE MAILED: 03/07/2017

**Determination of Patent Term Adjustment under 35 U.S.C. 154 (b)**  
 (Applications filed on or after May 29, 2000)

The Office has discontinued providing a Patent Term Adjustment (PTA) calculation with the Notice of Allowance.

Section 1(h)(2) of the AIA Technical Corrections Act amended 35 U.S.C. 154(b)(3)(B)(i) to eliminate the requirement that the Office provide a patent term adjustment determination with the notice of allowance. See Revisions to Patent Term Adjustment, 78 Fed. Reg. 19416, 19417 (Apr. 1, 2013). Therefore, the Office is no longer providing an initial patent term adjustment determination with the notice of allowance. The Office will continue to provide a patent term adjustment determination with the Issue Notification Letter that is mailed to applicant approximately three weeks prior to the issue date of the patent, and will include the patent term adjustment on the patent. Any request for reconsideration of the patent term adjustment determination (or reinstatement of patent term adjustment) should follow the process outlined in 37 CFR 1.705.

Any questions regarding the Patent Term Extension or Adjustment determination should be directed to the Office of Patent Legal Administration at (571)-272-7702. Questions relating to issue and publication fee payments should be directed to the Customer Service Center of the Office of Patent Publication at 1-(888)-786-0101 or (571)-272-4200.

## OMB Clearance and PRA Burden Statement for PTOL-85 Part B

The Paperwork Reduction Act (PRA) of 1995 requires Federal agencies to obtain Office of Management and Budget approval before requesting most types of information from the public. When OMB approves an agency request to collect information from the public, OMB (i) provides a valid OMB Control Number and expiration date for the agency to display on the instrument that will be used to collect the information and (ii) requires the agency to inform the public about the OMB Control Number's legal significance in accordance with 5 CFR 1320.5(b).

The information collected by PTOL-85 Part B is required by 37 CFR 1.311. The information is required to obtain or retain a benefit by the public which is to file (and by the USPTO to process) an application. Confidentiality is governed by 35 U.S.C. 122 and 37 CFR 1.14. This collection is estimated to take 12 minutes to complete, including gathering, preparing, and submitting the completed application form to the USPTO. Time will vary depending upon the individual case. Any comments on the amount of time you require to complete this form and/or suggestions for reducing this burden, should be sent to the Chief Information Officer, U.S. Patent and Trademark Office, U.S. Department of Commerce, P.O. Box 1450, Alexandria, Virginia 22313-1450. DO NOT SEND FEES OR COMPLETED FORMS TO THIS ADDRESS. SEND TO: Commissioner for Patents, P.O. Box 1450, Alexandria, Virginia 22313-1450. Under the Paperwork Reduction Act of 1995, no persons are required to respond to a collection of information unless it displays a valid OMB control number.

## Privacy Act Statement

**The Privacy Act of 1974 (P.L. 93-579)** requires that you be given certain information in connection with your submission of the attached form related to a patent application or patent. Accordingly, pursuant to the requirements of the Act, please be advised that: (1) the general authority for the collection of this information is 35 U.S.C. 2(b)(2); (2) furnishing of the information solicited is voluntary; and (3) the principal purpose for which the information is used by the U.S. Patent and Trademark Office is to process and/or examine your submission related to a patent application or patent. If you do not furnish the requested information, the U.S. Patent and Trademark Office may not be able to process and/or examine your submission, which may result in termination of proceedings or abandonment of the application or expiration of the patent.

The information provided by you in this form will be subject to the following routine uses:

1. The information on this form will be treated confidentially to the extent allowed under the Freedom of Information Act (5 U.S.C. 552) and the Privacy Act (5 U.S.C. 552a). Records from this system of records may be disclosed to the Department of Justice to determine whether disclosure of these records is required by the Freedom of Information Act.
2. A record from this system of records may be disclosed, as a routine use, in the course of presenting evidence to a court, magistrate, or administrative tribunal, including disclosures to opposing counsel in the course of settlement negotiations.
3. A record in this system of records may be disclosed, as a routine use, to a Member of Congress submitting a request involving an individual, to whom the record pertains, when the individual has requested assistance from the Member with respect to the subject matter of the record.
4. A record in this system of records may be disclosed, as a routine use, to a contractor of the Agency having need for the information in order to perform a contract. Recipients of information shall be required to comply with the requirements of the Privacy Act of 1974, as amended, pursuant to 5 U.S.C. 552a(m).
5. A record related to an International Application filed under the Patent Cooperation Treaty in this system of records may be disclosed, as a routine use, to the International Bureau of the World Intellectual Property Organization, pursuant to the Patent Cooperation Treaty.
6. A record in this system of records may be disclosed, as a routine use, to another federal agency for purposes of National Security review (35 U.S.C. 181) and for review pursuant to the Atomic Energy Act (42 U.S.C. 218(c)).
7. A record from this system of records may be disclosed, as a routine use, to the Administrator, General Services, or his/her designee, during an inspection of records conducted by GSA as part of that agency's responsibility to recommend improvements in records management practices and programs, under authority of 44 U.S.C. 2904 and 2906. Such disclosure shall be made in accordance with the GSA regulations governing inspection of records for this purpose, and any other relevant (i.e., GSA or Commerce) directive. Such disclosure shall not be used to make determinations about individuals.
8. A record from this system of records may be disclosed, as a routine use, to the public after either publication of the application pursuant to 35 U.S.C. 122(b) or issuance of a patent pursuant to 35 U.S.C. 151. Further, a record may be disclosed, subject to the limitations of 37 CFR 1.14, as a routine use, to the public if the record was filed in an application which became abandoned or in which the proceedings were terminated and which application is referenced by either a published application, an application open to public inspection or an issued patent.
9. A record from this system of records may be disclosed, as a routine use, to a Federal, State, or local law enforcement agency, if the USPTO becomes aware of a violation or potential violation of law or regulation.

<b><i>Examiner-Initiated Interview Summary</i></b>	<b>Application No.</b>	<b>Applicant(s)</b>	
	14/223,249	BARD ET AL.	
	<b>Examiner</b>	<b>Art Unit</b>	
	MYKOLA V. KOVALENKO	1662	

All participants (applicant, applicant's representative, PTO personnel):

(1) MYKOLA V. KOVALENKO. (3) \_\_\_\_.

(2) ELIZABETH LEHR STETZER. (4) \_\_\_\_.

Date of Interview: 09 February 2017.

Type: ☒ Telephonic ☐ Video Conference  
☐ Personal [copy given to: ☐ applicant ☐ applicant's representative]

Exhibit shown or demonstration conducted: ☐ Yes ☒ No.  
If Yes, brief description: \_\_\_\_.

Issues Discussed ☐101 ☒112 ☐102 ☐103 ☒Others  
(For each of the checked box(es) above, please describe below the issue and detailed description of the discussion)

Claim(s) discussed: All pending.

Identification of prior art discussed: \_\_\_\_.

**Substance of Interview**  
(For each issue discussed, provide a detailed description and indicate if agreement was reached. Some topics may include: identification or clarification of a reference or a portion thereof, claim interpretation, proposed amendments, arguments of any applied references etc...)

The Examiner and Applicant's representative agreed on the amendments to the claims that would put the application in condition for allowance.

**Applicant recordation instructions:** It is not necessary for applicant to provide a separate record of the substance of interview.

**Examiner recordation instructions:** Examiners must summarize the substance of any interview of record. A complete and proper recordation of the substance of an interview should include the items listed in MPEP 713.04 for complete and proper recordation including the identification of the general thrust of each argument or issue discussed, a general indication of any other pertinent matters discussed regarding patentability and the general results or outcome of the interview, to include an indication as to whether or not agreement was reached on the issues raised.

☐ Attachment

/MYKOLA V. KOVALENKO/ Primary Examiner, Art Unit 1662	
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<b>Notice of Allowability</b>	<b>Application No.</b> 14/223,249	<b>Applicant(s)</b> BARD ET AL.	
	<b>Examiner</b> MYKOLA V. KOVALENKO	<b>Art Unit</b> 1662	<b>AIA (First Inventor to File) Status</b> No

**-- The MAILING DATE of this communication appears on the cover sheet with the correspondence address--**

All claims being allowable, PROSECUTION ON THE MERITS IS (OR REMAINS) CLOSED in this application. If not included herewith (or previously mailed), a Notice of Allowance (PTOL-85) or other appropriate communication will be mailed in due course. **THIS NOTICE OF ALLOWABILITY IS NOT A GRANT OF PATENT RIGHTS.** This application is subject to withdrawal from issue at the initiative of the Office or upon petition by the applicant. See 37 CFR 1.313 and MPEP 1308.

1. ☒ This communication is responsive to Amendments and Remarks filed on December 8, 2016.  
☐ A declaration(s)/affidavit(s) under **37 CFR 1.130(b)** was/were filed on \_\_\_\_\_.
2. ☐ An election was made by the applicant in response to a restriction requirement set forth during the interview on \_\_\_\_\_; the restriction requirement and election have been incorporated into this action.
3. ☒ The allowed claim(s) is/are 1-4. As a result of the allowed claim(s), you may be eligible to benefit from the **Patent Prosecution Highway** program at a participating intellectual property office for the corresponding application. For more information, please see [http://www.uspto.gov/patents/init\\_events/pph/index.jsp](http://www.uspto.gov/patents/init_events/pph/index.jsp) or send an inquiry to PPHfeedback@uspto.gov.
4. ☐ Acknowledgment is made of a claim for foreign priority under 35 U.S.C. § 119(a)-(d) or (f).

**Certified copies:**

a) ☐ All    b) ☐ Some    \*c) ☐ None of the:

1. ☐ Certified copies of the priority documents have been received.
2. ☐ Certified copies of the priority documents have been received in Application No. \_\_\_\_\_.
3. ☐ Copies of the certified copies of the priority documents have been received in this national stage application from the International Bureau (PCT Rule 17.2(a)).

\* Certified copies not received: \_\_\_\_\_.

Applicant has THREE MONTHS FROM THE "MAILING DATE" of this communication to file a reply complying with the requirements noted below. Failure to timely comply will result in ABANDONMENT of this application.  
**THIS THREE-MONTH PERIOD IS NOT EXTENDABLE.**

5. ☐ CORRECTED DRAWINGS ( as "replacement sheets") must be submitted.  
☐ including changes required by the attached Examiner's Amendment / Comment or in the Office action of Paper No./Mail Date \_\_\_\_\_.

**Identifying indicia such as the application number (see 37 CFR 1.84(c)) should be written on the drawings in the front (not the back) of each sheet. Replacement sheet(s) should be labeled as such in the header according to 37 CFR 1.121(d).**

6. ☐ DEPOSIT OF and/or INFORMATION about the deposit of BIOLOGICAL MATERIAL must be submitted. Note the attached Examiner's comment regarding REQUIREMENT FOR THE DEPOSIT OF BIOLOGICAL MATERIAL.

**Attachment(s)**

1. <input type="checkbox"/> Notice of References Cited (PTO-892) 2. <input checked="" type="checkbox"/> Information Disclosure Statements (PTO/SB/08), Paper No./Mail Date _____ 3. <input type="checkbox"/> Examiner's Comment Regarding Requirement for Deposit of Biological Material 4. <input checked="" type="checkbox"/> Interview Summary (PTO-413), Paper No./Mail Date _____	5. <input checked="" type="checkbox"/> Examiner's Amendment/Comment 6. <input checked="" type="checkbox"/> Examiner's Statement of Reasons for Allowance 7. <input type="checkbox"/> Other _____
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/MYKOLA V. KOVALENKO/ Primary Examiner, Art Unit 1662	
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Application/Control Number: 14/223,249  
Art Unit: 1662

Page 2

**EXAMINER'S COMMENT**

1. The present application is being examined under the pre-AIA first to invent provisions.
2. Claims 1-8 are pending.

***Terminal Disclaimer***

3. The terminal disclaimers filed on December 8, 2017 disclaiming the terminal portion of any patent granted on this application which would extend beyond the expiration date of co-pending application 14/234,923 and US Patent 8,680,363 have been reviewed and is accepted. The terminal disclaimers have been recorded.

***Examiner's Amendment***

4. An examiner's amendment to the record appears below. Should the changes and/or additions be unacceptable to applicant, an amendment may be filed as provided by 37 CFR 1.312. To ensure consideration of such an amendment, it MUST be submitted no later than the payment of the issue fee.

Authorization for this examiner's amendment was given in a telephone interview with Elizabeth Lehr Stetzer, Applicant's representative, on February 9 and February 14, 2017.

***In the claims***

5. The claims have been amended as follows:

Claims 5-8 have been canceled.

In claim 4, line 1, the phrase "The isolated polynucleotide in claim 3" has been amended to recite --An isolated polynucleotide--.

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 3

In claim 4, lines 2-5, each instance of the phrase “base pairs” has been replaced with the term --nucleotides--.

***Reasons for Allowance***

6. The following is an examiner’s statement of reasons for allowance:

The claims are free of the art due to the failure of the prior art to teach or reasonably suggest the full-length SEQ ID NO: 14, which is the nucleotide sequence of event 9582.814.19.1; the polynucleotides of the instant SEQ ID NO: 1 or 2; or the recited fragments of SEQ ID NO: 1 or 2 diagnostic for the event and comprising the junction.

Claims 1 and 2 are interpreted as requiring the full-length SEQ ID NO: 14. Claim 3 is interpreted as requiring the full-length SEQ ID NO: 1, SEQ ID NO: 2, or SEQ ID NO: 14.

Applicant's amendments to claims 2 and 3 obviate the rejections under 35 U.S.C. 112 first and second paragraphs. The rejections have been withdrawn.

Examiner’s amendments to claim 4, above, obviate the rejection under 35 U.S.C. 112 fourth paragraph. The rejection has been withdrawn.

The statutory double-patenting rejection of claim 2 under 35 U.S.C. 101 as claiming the same invention as claim 7 of US Patent 8,680,363 has been withdrawn in view of Applicant's amendments to claim 2.

The non-statutory double-patenting rejections of claims 1, 3, and 4, over claims 1-9 of US Patent 8,680,363; and the provisional non-statutory double patenting rejection of claims 3 and 4 over claims 6, 8, 9, and 13 of the co-pending application 14/234,923

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 4

have been withdrawn in view of Applicant's filing of the terminal disclaimers, which were approved on January 5, 2017.

The provisional non-statutory double patenting rejection of claims 3 and 4 over claim 12 of co-pending application 14/784,044 is withdrawn in view of Applicant's argument in the Remarks and upon further consideration.

Any comments considered necessary by applicant must be submitted no later than the payment of the issue fee and, to avoid processing delays, should preferably accompany the issue fee. Such submissions should be clearly labeled "Comments on Statement of Reasons for Allowance."

***Allowable Subject Matter***

7. Claims 1-4 are allowed.

***Contact Information***

8. Any inquiry concerning this communication or earlier communications from the examiner should be directed to MYKOLA V. KOVALENKO whose telephone number is (571) 272-6921. The examiner can normally be reached on Monday-Friday 9:00 am - 5:30 pm PST.

If attempts to reach the examiner by telephone are unsuccessful, the examiner's supervisor, ZHOU (JOE) SHUBO can be reached at (571) 272-0724. The fax phone number for the organization where this application or proceeding is assigned is 571-273-8300.

Application/Control Number: 14/223,249  
Art Unit: 1662

Page 5

Information regarding the status of an application may be obtained from the Patent Application Information Retrieval (PAIR) system. Status information for published applications may be obtained from either Private PAIR or Public PAIR. Status information for unpublished applications is available through Private PAIR only. For more information about the PAIR system, see <http://pair-direct.uspto.gov>. Should you have questions on access to the Private PAIR system, contact the Electronic Business Center (EBC) at 866-217-9197 (toll-free). If you would like assistance from a USPTO Customer Service Representative or access to the automated information system, call 800-786-9199 (IN USA OR CANADA) or 571-272-1000.

/MYKOLA V. KOVALENKO/  
Primary Examiner, Art Unit 1662